NITROGEN CYCLE IN FLOATING-RAFT AQUAPONIC SYSTEMS

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To farmers

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ABSTRACT

Aquaponics recycles nitrogen and produces organic crops (fish and vegetables/fruits) with simultaneous treatment of nitrogen-rich aquaculture wastewater. The nitrogen cycle in aquaponics relies on the symbiotic relationships among bacteria, fish, and plants. However, there is lack of clear understanding of microbial ecology and nitrogen transformations in aquaponics which limits a widespread adoption of aquaponic systems by farmers. Such science-based information is critically important to achieve high food productivity and nitrogen use efficiency (NUE) in aquaponic systems. This study elucidated the nitrogen transformations in floating-raft aquaponic systems using several techniques, such as nitrogen mass balance, natural abundance and enriched nitrogen stable isotope ratios, quantitative polymerase chain reaction (qPCR), and next-generation sequencing of 16S rRNA gene. The nitrogen transformations in the aquaponic systems were studied using tilapia (*Oreochromis* spp.) and four different plant species, namely pak choi (*Brassica rapa* L. *Chinensis*), lettuce (*Lactuca sativa longifolia* cv. *Jericho*), chive (*Allium schoenoprasum L.*), and tomato (*Lycopersicum esculentum*).

Hydraulic loading rate (HLR), dissolved oxygen (DO), and pH were found to be the critical operating parameters to maintain efficient nitrification, resulting in excellent water quality for fish, plants, and bacteria. DO levels were associated with HLR and found to positively affect nitrite oxidation rate. Nitrite concentration increased in recirculating water under low DO levels. Nitrite concentrations in the aquaponic systems at a steady state significantly increased by 1.8-2.1 times when HLRs decreased from 1.5 to 0.25 m^3/m^2-day, and total ammonia nitrogen (TAN) concentrations at a steady state significantly increased by 2.1 times when HLRs decreased from 1.5 to 0.10 m^3/m^2-day. Low pH levels (5.2-6.0) were a major factor that shifted the microbial communities and reduced the relative abundance of nitrifiers in aquaponic components (plant root and biofilter), leading to total ammonia nitrogen accumulation in recirculating water. Interestingly, in plant roots, the abundances of essential nitrifier, *Nitrospira* spp., did not decrease at low pH levels (pH 5.2), suggesting the benefit of plants in aquaponics for improving nitrogen recovery.

Nitrification and denitrification occurred simultaneously in the aquaponics, resulting in nitrogen loss (10.3-40.4% of nitrogen input, depending on feeding rate). Based on the isotope

studies, nitrate was a major source of nitrogen assimilated by plants. However, nitrite and nitrate were the major sources of nitrogen loss via denitrification. Denitrification via direct nitrite reduction (33.7-53.4%), which was enhanced by low DO levels, was found to occur simultaneously with complete denitrification from nitrate. Nitrogen loss via denitrification was reduced by 36.9% and 74.5% when the fish feed feeding rates were decreased by 30% and 70%, respectively. Moreover, with nitrogen loss, the aquaponic systems also emitted nitrous oxide (N_2O) gas, a potent greenhouse gas, accounting up to 0.72-1.03 % of the nitrogen input. Aquaponics without the balance between fish feed and plants decreased the nitrogen recovery efficiency and contributed high nitrogen loss via denitrification (under an anoxic condition) and N_2O emissions.

The better understanding of nitrogen cycle linking with the microbial community and operating parameters was helpful in developing guidelines for aquaponic growers to improve water quality and archive high productivity from aquaponic systems while reducing environmental problems and operating cost.

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LIST OF ABBREVIATIONS

*amo*A	Ammonia monooxygenase subunit A
ANNOVA	Analysis of variance
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
ANAMMOX	Anaerobic ammonium oxidation
C:N	Carbon to nitrogen
CCA	Canonical correspondence analysis
COD	Chemical oxygen demand
DO	Dissolved oxygen
DNA	Deoxyribonucleic acid
DTPA	Diethylenetriamine pentaacetic acid
EM	Effective microorganism
EIE	Equilibrium isotope effect
GC-ECD	Gas chromatography - electron capture detector
HLR	Hydraulic loading rate
KIE	Kinetic isotope effect
NFT	Nutrient film technique
N	Nitrogen
N_2	Nitrogen gas (Dinitrogen)
NGS	Next-generation sequencing
NH_3	Free ammonia
NH_4^+	Ammonium
NH_2OH	Hydroxylamine
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	Nitrite-oxidizing bacteria
N_2O	Nitrous oxide
NOH	Nitroxyl

NUE	Nitrogen use recovery
OTU	Operational taxonomic units
OUR	Oxygen uptake rate
PCR	Polymerase chain reaction
PN	Partial nitrification
qPCR	Quantitative polymerase chain reaction
RAS	Recirculating aquaculture system
SOUR	Specific oxygen uptake rate
TAN	Total ammonia nitrogen
TKN	Total Kjeldahl nitrogen
VSS	Volatile suspended solid
16S rRNA	Ribosomal ribonucleic acid subunit 16

CHAPTER 1

INTRODUCTION

1.1. Background

Rapid growth in food demand due to increasing global population coupled with rising affluence will have significant impacts on food security. The consumption rates of food and nitrogen-rich fertilizer use are projected to outpace their availability by 2050 and 2018, respectively (Conforti, 2009; FAO, 2015). Rapid growth of nitrogen use in agro-food production systems has been a major concern of reactive nitrogen released into the environment (Matassa et al., 2015; Pikaar et al., 2017; Zhang et al., 2015). Without an improvement in nitrogen recovery, global nitrogen input in food production is projected to increase from 174 teragram (Tg) N/year in 2010 to 255 Tg N/year in 2050 due to an increase in world population to approximately 9.7 billion by 2050 (FAO, 2016; Zhang et al., 2015). Reactive nitrogen (e.g., ammonia and nitrate) loss from agro-food production can inevitably release into the environment via leaching, infiltration, or volatilization to the environments, which accounted for nearly 100 Tg N/year in 2010 (Zhang et al., 2015). The higher reactive nitrogen loss relative to the nitrogen use has severely impacted the environments via biogeochemical pathways, such as eutrophication of surface water, nitrate contamination of groundwater, and emission of nitrous oxide (N_2O) into the atmosphere (Matassa et al., 2015; Pikaar et al., 2017; Zhang et al., 2015). The growing food insecurity, uncontrollable rising of food prices, water scarcity and poverty, especially in developing countries, coupled with concerns of extreme changes in climate pattern are significant global challenges (Beddington et al., 2012; FAO, 2011; IPCC, 2013; Mukuve and Fenner, 2015; Seneviratne et al., 2012).

To overcome these issues, agriculture-based sustainable farming should produce food by conserving water, recycling nutrients, and converting wastes and wastewater into high-value resources (Beddington et al., 2012). For example, recovery of nitrogen input as products, defined as nitrogen use efficiency (NUE), must be improved from 40% in 2015 to 70% in 2050 (Zhang et al., 2015). To improve NUE, industrial farming can be integrated with other alternative systems (e.g., microbial protein from ammonia nitrogen, struvite production from wastewater, and

recirculating aquaculture systems among others) to reduce both nitrogen inputs for food production and nitrogen loss (Matassa et al., 2015; Pikaar et al., 2017; Zhang et al., 2015).

Importantly, with diminishing capture fisheries and increasing demand for healthy protein sources, aquaculture has grown rapidly at an annual rate of 7.9% with a global market of 148 billion US dollars in 2014 (FAO, 2016). The rapid growth of aquaculture, however, has several issues such as large land requirement and high water demand, and environmental concerns (FAO, 2016; Hu et al., 2012). Therefore, the expansion of world aquaculture requires new technologies to intensify fish production while maximizing nutrient recovery and minimizing the environmental impacts associated with the uncontrolled discharge of nutrient-rich wastewater (FAO, 2016; Hu et al., 2015).

Aquaponics, a soilless crop production system that integrates both aquaculture production and soilless agriculture could play an important role in nitrogen recovery from intensive aquaculture wastewater (effluent) to organically-grown vegetables (Wongkiew et al., 2017a, 2017b). Aquaponic system offers several other merits such as simplicity, zero effluent discharge, recovery of high-value fish and vegetables/fruits, and high nutrient availability for plants over other nitrogen recovery technologies (Cornejo et al., 2016; Engle, 2015; Love et al., 2015; Wongkiew et al., 2017a, 2017b). Aquaponic systems have also been successfully commercialized for nitrogen recovery from freshwater aquaculture systems with 11-28% annual net return on investment, and high flexibility for investment and scale-up (Engle, 2015; David C. Love et al., 2015). Therefore, aquaponics has great potential to become a sustainable technology for nitrogen-rich wastewater remediation with the simultaneous year-round production of high-quality fish and vegetables while conserving the water.

However, due to the simultaneous operation of aquaculture and hydroponic systems, it is often challenging to maximize both fish production and nitrogen recovery by plants in large-scale aquaponics. There have been several attempts to optimize the performances of aquaponic systems, such as balancing plant uptake and fish output, and recovering nutrients (Buzby and Lin, 2014; Hu et al., 2015; Lam et al., 2015). However, these optimization studies were specific to particular design and operating conditions. For example, Endut et al. (2010) varied hydraulic loading rates (HLRs) to increase the nitrogen uptake and plant growth rates. However, few studies have focused on optimizing NUE, water quality, and operations. By combining nitrogen

mass balance and nitrogen isotope techniques, the nitrogen cycle in aquaponic systems can be elucidated (Casciotti and Buchwald, 2012; Ryabenko, 2013). Understanding of the key mechanisms of nitrogen transformations will help to design and operate an efficient aquaponic system. Thus, studying the nitrogen cycle and operating factors affecting nitrogen transformations are critically important to optimize aquaponic performances and improve overall NUE.

Nitrogen recovery in aquaponic systems relies on close linkage among bacteria, fish, and plants. In aquaponic systems, the excreted ammonium from fish must be oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), respectively, to avoid ammonium and nitrite toxicity. Nitrate is assimilated by plants in aquaponic systems thereby increasing the NUE (Öhlund and Näsholm, 2002; Wongkiew et al., 2017a, 2017b). In ammonia and nitrite oxidations, pH in recirculating water decreases due to nitrification (Pynaert et al., 2004; Wongkiew et al., 2017a). Low pH also affects AOB and NOB, reduces nitrification efficiency, stresses fish, and enhances N_2O emission (Hou et al., 2017; Hu et al., 2012; Liu et al., 2016; Zou et al., 2016b). Therefore, an efficient aquaponic system with high NUE and high nitrification rates could be achieved by maintaining adequate concentrations and activities of nitrifying and denitrifying bacteria (Cabrol et al., 2016).

There are very few studies that employed molecular techniques to evaluate the link between microbial communities and nitrogen transformations in aquaponic systems, which is critically essential to overcome the limitation of improving NUE. Using quantitative polymerase chain reaction (qPCR), for example, Hu et al. (2015) and Zou et al. (2016b) suggested that pH of 6.0 with tomato as a plant species in aquaponic systems resulted in high relative abundances of genes encoding ammonia monooxygenase subunit A (*amo*A), nitrite reductases (*nir*S and *nir*K), and nitrous oxide reductase (*nos*Z). However, qPCR is very specific to targeted microbial groups (Ye et al., 2012). As a result, other microbial groups contributing to nitrogen transformations are still unknown in an aquaponic system. Recently, Schmautz et al. (2017) employed next-generation sequencing (NGS) and metagenomics to evaluate the microbial communities in aquaponic compartments including plant roots, biofilters, periphyton, and fish feces in a well-operated aquaponic system. Several studies reported the occurrence of biases during the sequencing and alignment when performing NGS (Ju and Zhang, 2015; Park et al., 2017). Thus,

there is a need to evaluate microbial community for different plant species and pH levels in aquaponic systems using a combination of qPCR and NGS to meticulously examine the ecology and interactions of important microorganisms, and their effects on the performance of aquaponic systems (e.g., NUE and nitrification rate) (Ju and Zhang, 2015; Ye et al., 2012).

In spite of several benefits of aquaponic systems, it emits N_2O (Hu et al., 2015). N_2O is an atmospheric greenhouse gas that has nearly 300 times higher potential to contribute to global warming than CO_2 on per molecule basis (Forster et al., 2007). Atmospheric N_2O also plays a primary role in ozone depletion in the stratosphere (Ravishankara et al., 2009). N_2O in aquaponic systems can be produced by nitrification and denitrification (Buzby and Lin, 2014; Hu et al., 2015). There are few studies on N_2O emissions from aquaponic systems with a particular emphasis on reducing N_2O emission (Hu et al., 2015; Jia et al., 2013; Zou et al., 2016b). For example, Zou et al. (2016) and Fang et al. (2017) focused on the optimum pH and aeration pattern for reducing N_2O emission in media-based aquaponics. However, studies on other types and plant species of aquaponic systems, and other mitigation measures to reduce N_2O emissions are still lacking. Such information will be helpful for aquaponic growers to reduce N_2O emissions and manage the nitrogen and products from aquaponic systems more efficiently.

1.2. Objectives of the study

The overarching goal of this research is to obtain science-based information on nitrogen transformations in aquaponic systems. The specific objectives of this study are:

(1) quantify the impact of physical and chemical variables that regulate nitrogen transformations in aquaponic systems.

(2) examine the transformations of different forms of nitrogen in an aquaponic system under different conditions.

(3) examine the ecology of functionally important microbes and assess their contributions to nitrogen transformations in aquaponic systems.

(4) investigate the greenhouse gas emissions from an aquaponic system, with specific emphasis on N_2O emission.

1.3. Scope of the study

This study evaluated the nitrogen cycle in pilot-scale floating-raft aquaponic systems with four plant species, namely lettuce, pak choi, tomato, and chive. Tilapia was the growing fish species in the aquaponic systems. This study evaluated the effect of dissolved oxygen (DO), HLR, and pH on nitrogen transformations in the aquaponic systems. The ecology of the bacterial community in the aquaponic systems was examined using 16S rRNA gene sequencing and qPCR. Adding effective microorganism (EM) and supplying air to biofilters were two proposed strategies to reduce N_2O emissions from aquaponic systems in this study. This study was conducted to elucidate the understanding of the nitrogen cycle in the aquaponic systems for guiding aquaponic farmers and developers to improve the performance and nitrogen recovery of aquaponic systems. This study, however, did not focus on maximizing fish and plant growths or N_2O emission reduction.

CHAPTER 2

LITERATURE REVIEW

2.1. Aquaponic systems

2.1.1. What is aquaponics?

Aquaponics is a soilless agriculture system that synergistically combines a recirculating aquaculture system (RAS) with hydroponics (Love et al., 2015). In aquaponic systems, fish excrete nutrient-rich waste as part of their metabolism especially in the form of ammonium nitrogen and other nutrients into the aqueous phase in aquaculture tanks. As the aquaculture effluent flows into the hydroponic component, nutrients are transformed by diverse microbial communities (e.g., nitrifying bacteria, organotrophic bacteria) and assimilated by plants in the form of inorganic compounds (e.g., nitrate, phosphate) (Graber and Junge, 2009; Zou et al., 2016b). Figure 2.1 shows the schematic diagram of the symbiotic relationship among fish, microbial community, and plants in a floating-raft aquaponic system. The main merits of aquaponic systems are recovering nutrients, minimizing water demand, and increasing profitability by simultaneously producing two cash crops (Love et al., 2015).

Aquaponics combines the advantages of both aquaculture and hydroponics while eliminating their disadvantages (e.g., cost of remediating nitrogen-rich effluent from aquaculture and cost of supplementing nutrient solutions in hydroponics). Aquaponics also reduces the operating costs when considering each system individually (Love et al., 2015). Aquaponics has been emerging as a sustainable farming method in providing year round high quality fish and vegetables while conserving water.

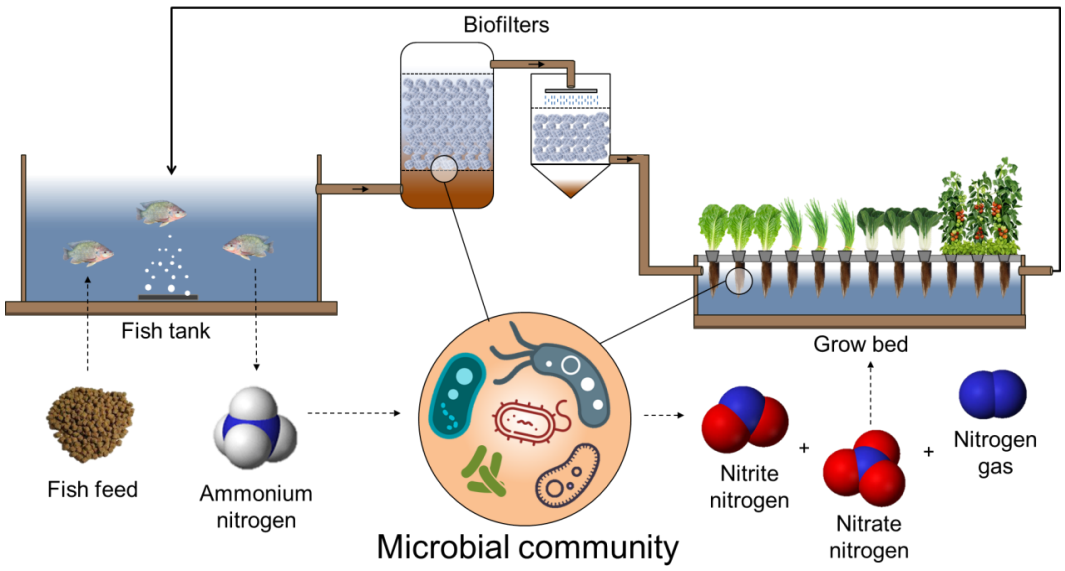

Figure 2.1. Schematic diagram of the symbiotic relationship among fish, microbial community, and plants in an aquaponic system

2.1.2. Types of aquaponic systems

A typical aquaponic system consists of a fish tank (aquaculture), a biofilter (for nitrification), and a grow bed (hydroponics) (Love et al., 2015). There are three types of most commonly used aquaponic systems (Figure 2.2), classified based on types of grow bed, namely nutrient film technique (NFT), floating-raft (deep water culture) and media-filled (flood and drain) (Engle, 2015). NFT type provides high oxygen to the plant roots that facilitates high yield of vegetables. However, NFT is only suitable for small vegetable species because the grow bed cannot support a high quantity of roots due to potential blockage of recirculating flow (Chérif et al., 1997; Engle, 2015). Thus, efficient solid removal is critical for NFT to prevent the clogging in the grow bed channel. Floating-raft type is the most commonly adopted aquaponic system because it allows the plant roots to freely absorb the nutrients in the water without clogging the water channel (Engle, 2015; Liang and Chien, 2013; Timmons et al., 2002). NFT and floating-raft aquaponic systems require a biofilter for nitrification and a sedimentation tank for solid removal (Engle, 2015; Nelson, 2008). Media-filled (or flood-and-drain) type is the simplest aquaponic system that does not require separate biofilters because the media-filled aquaponics contains media (e.g., pumice stones or clay beads) in the grow bed for nitrification (Zou et al.,

2016b). A siphon is used to fill and drain the water in order to supply oxygen by direct contact between plant roots and the air (Bernstein, 2011). However, clogging and insufficient oxygen levels commonly occur in the grow bed during long-term operation of this type of aquaponic system.

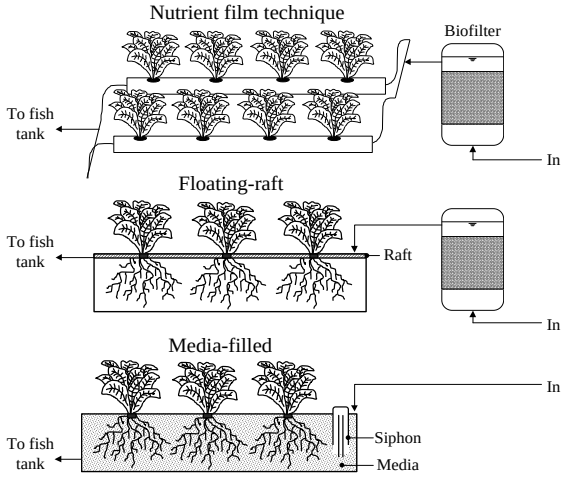

Figure 2.2. Aquaponic systems based on types of grow bed (Wongkiew et al., 2017a)

2.2. Transformations of nitrogen in aquaponic systems

2.2.1. Total ammonia nitrogen (TAN) excretion from fish

In fresh water, fish release ammonium (NH_4^+) through transamination and deamination process of feed protein in the liver. Excess L-amino acids derived from the protein are first transformed to glutamate by the catalytic reaction with alpha-ketoglutarate. Glutamate dehydrogenase enzyme then deaminates the glutamate to ammonium. This biochemical pathway regenerates alpha-ketoglutarate enzyme as the product, which is reused in the same cycle. The produced NH_4^+ and free ammonia (NH_3) molecules appear in the bloodstream of fish and partially accumulate in white muscle depending on electrochemical gradients between the bloodstream and the amount of white muscle (Wilkie, 1997). Since it is very difficult for the ionized NH_4^+ to permeate across the phospholipid of biological membranes from the bloodstream, passive unionized NH_3 diffusion drives the excretion of ammonia nitrogen to the bulk water through the branchial epithelium of fish (Ip and Chew, 2010; Wilkie, 2002). The high partial pressure of NH_3 in blood drives the NH_3 to permeates through gill epithelium via aqueous

pores (aquaporins) and paracellular routes, diffuses to gill water (unstirred boundary layer), and bulk water, respectively. Figure 2.3 (left box) shows the pathways of TAN generation by fish. At the gill water layer, the passing NH_3 will be protonated to NH_4^+ by the dissociation of dissolved CO_2, which is permeable within the gill, resulting in the release of NH_4^+ (Ip and Chew, 2010). CO_2 can also dissociate inside the gill cytosol by hydration reaction with carbonic anhydrase enzyme (CA). The released ammonia is then trapped with H^+, which is generated from the original dissolved CO_2 and the other H^+ ions. H^+ ion is actively excreted to apical side by the mediation of H^+-ATPase (Wilkie, 2002, 1997). Thus, the partial pressure of NH_3 in the blood and outside the gill is controlled by the gradients of NH_3 concentration and ambient pH, which equilibrates the fraction of NH_4^+ and NH_3. The sum of NH_4^+ and NH_3 is known as TAN; however, at the pH around neutral ($6.0 < pH < 7.2$), 100% of TAN dissociates and present in the form of NH_4^+ (see section 2.2.11). Figure 2.3 shows the nitrogen pathways in aquaponic systems.

Feed provides energy and nutrients for cell maintenance and assimilation (fish muscle growth), and free energy for metabolism (Lekang, 2013). The production rate of TAN can be approximated as given below (Ebeling et al., 2006; Timmons et al., 2002):

$$P_{TAN} = F \times PC \times 0.092 \qquad (2.1)$$

Where P_{TAN} is the production rate of TAN (kg/day); F is feeding rate (kg/day); PC is protein content (fraction); 0.092 is the fraction of excreted TAN per unit protein input. The feed consumption by adult fish is normally 1 to 3% of their body weight while juvenile fish consume around 7% of their body weight (Bernstein, 2011). In addition, feed conversion ratio (FCR) is defined as the ratio of the total weight of fish feed to total wet weight gained by fish at the end of the culture period (Endut et al., 2010). FCR value depends on fish species and feed type (Hu et al., 2014).

Aquaponic systems are generally operated using freshwater because the growth of plants and roots are stressed by osmotic pressure and are inhibited by high salinity (Duan et al., 2013). Ammonia excretion pathways in freshwater fish are different from those in marine fish due to their different mechanisms of osmoregulation. Freshwater fish (hypertonic) urinate to maintain a low blood concentration of solutes while marine fish (hypotonic) balance the high blood

concentration by drinking seawater and reducing the urine excretion (Evans, 2003; Weihrauch et al., 2009). Thus, freshwater fish provides a higher TAN generation rate than the fish in marine environment when feeding at the same rate (Ip and Chew, 2010).

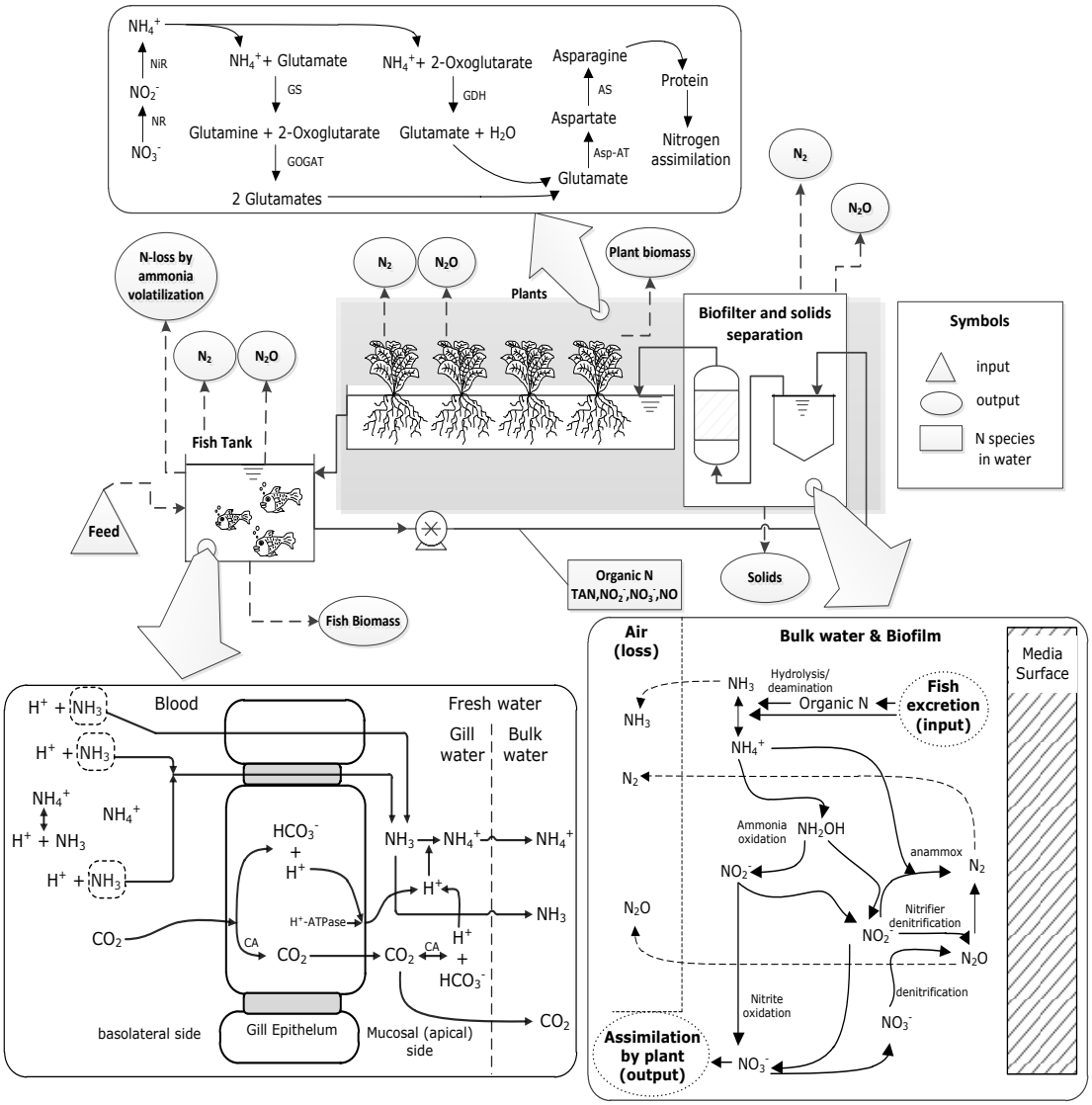

Figure 2.3. Schematic diagram of an aquaponic system and nitrogen transformations pathways in different components (Wongkiew et al., 2017a)

2.2.2. Hydrolysis of feces and suspended solids

The other pathway of TAN generation is the hydrolysis of organic N such as fish urine and feces. Urea hydrolysis occurs in biofilms of aquaponic biofilters. The hydrolysis rate can be approximated by first-order kinetics because urea concentrations in aquaponic systems are significantly lower than the half-saturation constant (Ks) (e.g., 33.1 g/L for *Escherichia coli* MJK2) of hydrolytic bacteria from Michaelis-Menten equation (Connolly et al., 2015). Fish excrete high amount of feces, and the microorganisms synthesize new cells in the presence of organic carbon (van Rijn, 2013). Total solids generations of about 230, 585, 448 and 224 kg/metric ton fish produced were reported in RAS for tilapia (*Oreochromis* sp.), Nile tilapia (*O. niloticus*), Gilthead seabream (*Sparus aurata*) and Atlantic salmon (*Salmo salar*), respectively (van Rijn, 2013). Nitrogen content in fish feces varies from 10 to 40% depending on a nitrogen content of fish feed and fish species (Lupatsch and Kissil, 1998; Schneider et al., 2004; van Rijn, 2013). The solids can be periodically removed by draining the sedimentation tank or biofilters.

Organic nitrogen compounds are hydrolyzed to ammonia nitrogen. The following equations show the hydrolysis of non-specific organic nitrogen, using R as a functional group of carbon (Eq. 2.2), and the hydrolysis of urea (Eq. 2.3).

$$R\text{-}NH_2 + 2H_2O \rightarrow NH_4^+ + R^+ + 2OH^- \qquad (2.2)$$

$$(H_2N)CO(NH_2) + H_2O \rightarrow 2NH_3 + CO_2 \qquad (2.3)$$

2.2.3. Nitrification

Nitrification is the main process that transforms ammonia nitrogen to nitrate in the presence of oxygen (Hu et al., 2015). TAN is oxidized to nitrite by ammonia-oxidizing bacteria (AOB) (e.g., *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosolobus, Nitrosovibrio* spp., *Nitrosocystis,* etc.) and ammonia-oxidizing archaea (AOA). The resulting nitrite is oxidized to nitrate by nitrite-oxidizing bacteria (NOB) (e.g., *Nitrobacter, Nitrococcus, Nitrospira, Nitrospina* spp., etc.) (Ebeling et al., 2006; Gerardi, 2002; Panuvatvanich et al., 2009). AOA do not appear to play a role in aquaponic systems although their abundance was reported in similar environments such as soils and oceans (Jung et al., 2014; Xia et al., 2011; Zhang et al., 2012).

AOA is responsible for oxidizing ammonia under extremely low NH_4^+ concentrations (about 2 µg N/L) due to their physiological diversity, leading to toleration and adaptation to extreme nutrient limiting conditions (Martens-Habbena et al., 2009). The nitrification rate of AOA will not be significantly higher than that of AOB in nitrogen-rich environments, such as aquaponic systems. Thus, nitrification by AOA does not play significant role in aquaponic systems (Hu et al., 2015; Zou et al., 2016b).

In essence, the fish tank and biofilters in aquaponic systems resemble RAS in terms of TAN and nitrite oxidation, which suggests that the same nitrifying microbial groups exist in both systems (Hu et al., 2015; Kuhn et al., 2010; Prehn et al., 2012). However, gene expressions of the microbial groups were also found on the surface of plant roots in aquaponic systems (Hu et al., 2015; Zou et al., 2016b). Using Fluorescence In Situ Hybridization (FISH) and 16S rRNA analysis, the abundance and activity of nitrifiers, such as *Nitrosomonas* spp., *Nitrococcus* spp., and *Nitrospira* spp., were observed in the biofilm of a moving bed biofilm reactor (MBBR) employed in a recirculating aquaculture system (van Kessel et al., 2010). AOB and NOB (*Nitrobacter* spp. and *Nitrospira* spp.) were found in aquaponic systems, particularly on the root surface of tomato (*Lycopersicon esculentum*) and pak choi (*Brassica campestris L. subsp. chinensis*) (Hu et al., 2015). AOB and NOB require oxygen as an electron acceptor for their metabolisms. The nitrifiers also utilized NH_3, which is deprotonated from NH_4^+ in the recirculating water of aquaponics, and inorganic carbon (dissolved carbon dioxide and bicarbonate) as nitrogen and carbon source, respectively for cell assimilation. In the biofilters treating ammonia-rich aquaculture effluent, the energy input is utilized in two electron accepting pathways: the synthesis of carbonaceous biomass (6.2%) and the generation of metabolized energy (93.8%) (Ebeling et al., 2006).

The two groups of chemoautotrophic bacteria (AOB and NOB) require oxygen as the electron acceptor for energy (Eq. 2.4). They assimilate carbon dioxide and bicarbonate as their carbon source and ammonium ions as nitrogen source (Eq. 2.5). Ammonium ion acts as an electron donor for energy in nitrification reaction (Eq. 2.6) (Rittmann & McCarty, 2001). During the nitrogen reduction activity, there are several unstable compounds such as hydroxylamine

(NH_2OH) and nitroxyl (NOH) as intermediates (Zaman et al., 2012). However, the concentrations of those compounds are relatively low in comparison to nitrite and nitrate.

$$0.25\ O_2 + H^+ + e^- \rightarrow 0.5\ H_2O \qquad (2.4)$$

$$0.20\ CO_2 + 0.05\ HCO_3^- + 0.05\ NH_4^+ + H^+ + e^- \rightarrow 0.05\ C_5H_7O_2N + 0.45\ H_2O \qquad (2.5)$$

$$0.125\ NH_4^+ + 0.375\ H_2O \rightarrow 0.125\ NO_3^- + 1.25\ H^+ + e^- \qquad (2.6)$$

To separate the activity of AOB and NOB from Eq. 2.6, ammonia and nitrite oxidations can be represented by Eqs. 2.7 and 2.8, respectively.

$$0.125\ NH_4^+ + 0.25\ H_2O \rightarrow 0.125\ NO_2^- + H^+ + 0.75e^- \qquad (2.7)$$

$$0.125\ NO_2^- + 0.125\ H_2O \rightarrow 0.125\ NO_3^- + 0.25\ H^+ + 0.25e^- \qquad (2.8)$$

To balance and combine the three reduction equations above, two pathways of energy (energy for cell activities and biomass production) taken from the electron donors have to be partially divided into two fractions: R_c and R_a (Eq. 2.9). Based on the assumption that energy-transfer efficiency is 0.6, and pyruvate is the intermediate of the biosynthesis, the mole fractions of cell synthesis (R_c) and energy utilization (R_a) can be represented by (Eq. 2.9). In Eq. 2.9, R_d, R_c, and R_a represent half-reaction for electron donor (100%), the synthesis of carbonaceous biomass (6.2%) and electron acceptor for energy (93.8%), respectively (Rittmann & McCarty, 2001). Thus, the reactions from Eqs. 2.4, 2.5, and 2.6 can be substituted to Eq. 2.9, and overall nitrification can be shown by Eq. 2.10. Since nitrifiers consume carbon dioxide dissolved in water (bicarbonate), equation Eq. 2.10 can be rewritten as Eq. 2.11 in the form of bicarbonate utilization (Rittmann & McCarty, 2001).

Eqs. 2.9 to 2.11 show the summaries of ideal nitrification by assuming nitrogen loss as N_2O and N_2 do not occur. However, in fact, N_2O and N_2 emissions can occur via nitrifier-denitrification in which ammonia and nitrite nitrogen can release from the recirculating water to atmosphere during nitrification process. N_2O emission and nitrifier denitrification are discussed in sections 2.28 and 2.2.9. Nitrogen mass balance can be conducted and apply to these

stoichiometry later once the contributions of nitrogen losses via denitrification and nitrification denitrifiers are known.

$$R=R_d-0.062*R_c-0.938*R_a \qquad (2.9)$$

$$NH_4^+ + 1.83\ O_2 + 0.094\ CO_2 + 0.024\ HCO_3^- \rightarrow 0.024\ C_5H_7O_2N + 0.977\ NO_3^- + 0.953\ H_2O + 1.95\ H^+ \quad (2.10)$$

$$NH_4^+ + 1.83\ O_2 + 1.97\ HCO_3^- \rightarrow 0.0244\ C_5H_7O_2N + 0.976\ NO_3^- + 2.90\ H_20 + 1.86\ CO_2 \qquad (2.11)$$

pH has to be controlled around 7.0 in aquaponic systems to maintain high nitrification efficiency due to the release of protons (Eq.2.11). However, studies have shown that nitrification rate decreased by as much as 90% at the pH around 6.0, compared to the nitrification rate at the optimum pH of 7.2 to 8.0 (Gerardi, 2002). Summary of nitrification, denitrification, and anammox, which are explained later, can be shown in the following nitrogen cycle (Figure 2.4).

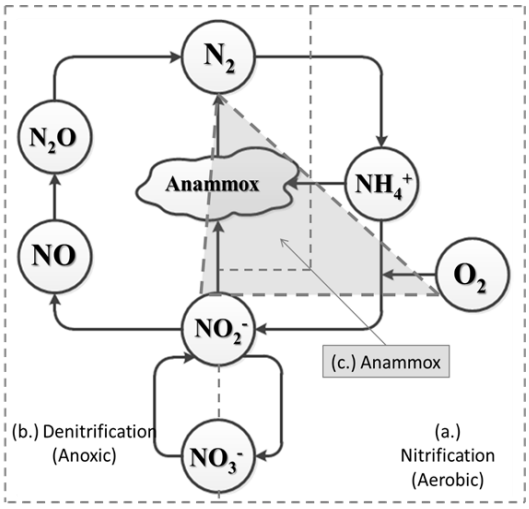

Figure 2.4. Nitrogen cycle and three main processes in aqueous phase: nitrification (a), denitrification (b.) and anammox (c.)

2.2.4. Denitrification

Denitrification occurs under low oxygen concentration (0.1-0.2 mg/L). In aquaponic systems, an anoxic condition occurs in biofilters and sedimentation tanks where a high concentration of suspended solids is accumulated (Hu et al., 2015). Denitrification is an biochemical process in aquaponic systems. Denitrification is carried out by various archaea and

facultative heterotrophic bacteria such as *Achromobacter*, *Aerobacter*, *Acinetobacter*, *Bacillus*, *Brevibacterium*, *Flavabacterium*, *Pseudomonas*, *Protus*, and *Micrococcus* spp. (Gentile et al., 2007; Hargreaves, 1998; Lu et al., 2014a; Michaud et al., 2006). These microbes are called denitrifiers. Denitrifiers use nitrate as an electron acceptor and utilize dissolved organic carbon in recirculating water as an electron donor. Denitrifiers transform nitrate to nitrite, nitric oxide (NO), nitrous oxide (N_2O), and finally to nitrogen gas (N_2) under anoxic condition (Eq. 2.12). Table 1 shows the genera of denitrifying bacteria.

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (2.12)$$

Table 2.1. Genera of bacteria in denitrifying process

Genera of bacteria in denitrification			
Acetobacter	*Bacillus*	*Halobacterium*	*Propionicbacterium*
Achromobacter	*Chromobacterium*	*Hyphomicrobium*	*Pseudomonas*
Acinetobacter	*Corynebacterium*	*Kingella*	*Rhizobium*
Agrobacterium	*Denitrobacillus*	*Methanonas*	*Rhodopseudomonas*
Alcaligenes	*Enterobacter*	*Moraxella*	*Spirillum*
Arthrobacter	*Escherichia*	*Neisseria*	*Thiobacillus*
Axotobacter	*Flavobacterium*	*Paracoccus*	*Xanthomonas*

Source: Gerardi (2002)

The kinetics of denitrification depends on available carbon source, pH, and the concentrations of each nitrogen species that could cause substrate or product inhibition. Carbon limitation affects the activity of denitrifying bacteria, which causes the accumulation of intermediate product, such as NO and N_2O, and also results in nitrate reduction to ammonium via the dissimilatory nitrate reduction to ammonia (DNRA) process (Hu et al., 2014; van Rijn et al., 2006). Thus, denitrification in aquaponic systems could be reduced by maintaining sufficient organic carbon and avoiding anoxic zone. Some heterotrophs, such as *Alcaligenes faecalis*, *Rhodococcus* spp. CPZ24, *Pseudomonas* spp., *Xanthomonadaceae* spp., and *Sphingomonas* spp., perform heterotrophic nitrification and denitrification simultaneously at low dissolved oxygen (DO) condition (< 0.3 mg/L) (P. Chen et al., 2012; Fitzgerald et al., 2015; Zhao et al., 2012).

Rhodococcus spp. CPZ24 was reported to transform 85% of ammonia nitrogen to nitrate (13%), biomass (24%), and gaseous nitrogen (48%) (P. Chen et al., 2012).

2.2.5. Nitrogen assimilation by microbes

Heterotrophic aerobic bacteria co-exist with nitrifiers and become dominant when organic carbon concentration or C:N ratio increases, leading to the generation of a high quantity of excess microbial biomass in the form of sediment (J.P. Blancheton et al., 2013; Michaud et al., 2014; Zhou et al., 2009). Heterotrophs utilize ammonium, nitrate, and organic carbon for cell growth. Hence, they will be more dominant than the autotrophs when organic carbon is available due to the higher growth rate of heterotrophs relative to autotrophs (~ 5-folds) (Díaz et al., 2012; Ling and Chen, 2005). Table 2.2 shows the common genera of heterotrophs that could be found in aquaponic systems and other ecosystems.

Table 2.2. Common genera of heterotrophs in aqueous environments

Genus	Strict Aerobes	Facultative Anaerobes
Achromobacter		x
Acinetobacter	x	
Actinomyces		x
Aerobacter		x
Arthrobacter	x	
Bacillus		x
Beggiatoa		x
Cornynebacterium		x
Enterobacter		x
Escherichia		x
Flavobacterium		x
Klebsiella		x
Micrococcus	x	
Norcardia	x	
Proteus		x
Pseudomonas		x
Sphaerotilus		x
Thiothrix	x	
Zooglea		x

Source: Gerardi (2002)

Heterotrophic bacterial growth can be explained using glucose as an electron donor (Eq. 2.13), oxygen is the electron acceptor (Eq. 2.4), and organic carbon for cell synthesis (Eq. 2.5).

Fractions of the electron for cell synthesis and energy utilization can be shown by (Eq. 2.14). 70% of the electron contributes to cell synthesis, and 30% of the electron contributes to energy (Rittmann & McCarty, 2001). This means that heterotrophs consume about 70% of the internal energy from ammonium generated by the fish for cell synthesis, while 30% of the remaining energy is used for cell metabolisms (Ebeling et al., 2006). This suggests that the cell yield of heterotrophic bacteria is higher than that of autotrophic nitrifying bacteria; contributing to a large amount of microbial biomass production in aquaponic systems when chemical oxygen demand (COD) is high. The low energy needed for the synthesis using carbonaceous compounds as an electron donor is attributed to the high growth rate of heterotrophs.

$$0.0417\ C_6H_{12}O_6 + 0.25\ H_2O \rightarrow 0.25\ CO_2 + H^+ + e^- \quad (2.13)$$

$$R=R_d - 0.70*R_c - 0.30*R_a \quad (2.14)$$

Eq. 2.15 shows an overall reaction of cell growth using ammonia nitrogen as nitrogen source by heterotroph aerobic bacteria.

$$NH_4^+ + 1.18\ C_6H_{12}O_6 + HCO_3^- + 2.06O_2 \rightarrow C_5H_7O_2N + 6.06\ H_2O + 3.07\ CO_2 \quad (2.15)$$

Equations (Eqs. 2.14 and 2.15) suggest that cell yield of heterotrophic bacteria is higher than autotrophic nitrifying bacteria. Although in realistic cases NH_4^+ and glucose are not the sole nitrogen and carbon sources in the recirculating water of aquaponics, these equations simplify the cell assimilation model at a molecular level where ideal substrates are available. The low energy requirement (high negative $\Delta G^{o'}$, Gibbs free energy) of the synthesis using glucose as an electron donor could be the reason why heterotrophic bacteria grow faster and become more predominant over autotrophic bacteria when high organic carbon is available. Thus, the yield of the biomass of nitrifying bacteria (autotroph) is relatively low compared to heterotrophic bacteria.

In aquaponic systems, heterotrophs grow by consuming dissolved organic carbon of decayed biomass and fish excreta as a carbon source under aerobic condition. Heterotrophic biomass was reported to account for about 7% of fish feed in aquaculture systems (Hu et al., 2012). This value, however, depends on C:N ratio, nitrogen concentrations, organic matter, COD concentration, and operational conditions. In a system where heterotrophs and nitrifiers coexist,

the rates of nitrate production/TAN consumption were reduced by 24%, 56% and 73% at C:N ratios of 0.5, 1.0 and 1.5, respectively (Ebeling et al., 2006).

2.2.6. Anaerobic ammonia oxidation (anammox)

Anaerobic ammonium oxidation (anammox) is also likely to exist in aquaponic systems because the water characteristics are similar to those in aquaculture systems, where anammox process has been shown to occur (Lahav et al., 2009; Timmons et al., 2002; van Kessel et al., 2010; Zou et al., 2016b). Anammox is a shortcut process that removes ammonia nitrogen by being oxidized to nitrite, and then the ammonium and nitrite are transformed to nitrogen gas directly by anammox bacteria without the function of denitrifiers (Eq. 2.16). Anammox process can occur simultaneously with nitrification process at low DO conditions (Lahav et al., 2009; Ma et al., 2015; van Kessel et al., 2010). In biofilm-based wastewater treatment systems, AOB were found at the outer layer of the biofilms in biofilters treating aquaculture effluent, which had the similar characteristics as aquaponic systems (Hu et al., 2015; Zou et al., 2016b), and anammox bacteria were detected in the inner layer of the biofilms (the oxygen-depleted zone), suggesting the co-existence of AOB and anammox bacteria (Ma et al., 2015; Magrí et al., 2013; van Kessel et al., 2010). Partial aerobic oxidation or partial nitrification (PN) is essential to generate nitrite for anammox process. AOB are supposed to play a dominant role, but the activity of NOB should not be active since the formation of nitrate is not the substrate for anammox process.

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (2.16)$$

Anammox process is performed by chemoautotrophs such as *Plantomycetes*-phylum bacteria. There are five main anammox bacterial groups: *Anammoxoglobus*, *Brocadia*, *Jettenia* and *Kuenenea*, and *Scalindua*. Anammox bacteria were reported in biofilters treating freshwater aquaculture systems, marine water and sediments, freshwater, and agricultural sediments (Castine et al., 2012; Francis et al., 2007; Hu et al., 2011; Magrí et al., 2013; Tal et al., 2006). Nitrogen gas can be formed via anammox process under an anoxic condition in aquaponic biofilters.

2.2.7. Nitrogen uptake by plants

Nitrogen uptake is the main pathway of nitrogen recycling into vegetables in aquaponic systems. An efficient aquaponic system should show high yields of plant and fish biomass with low amount of nitrogen loss. The nitrogen transformations in vegetable biomass is affected by the surface area of plant roots and contact time (Buhmann and Papenbrock, 2013). Furthermore, plants could affect the microbial community, microbial functions, and interactions among microorganisms (Wang et al., 2015).

Nitrogen uptake rate is influenced by many factors such as nutrient concentrations, light intensity, humidity, temperature, and ambient carbon dioxide concentration (Tiaz and Zeiger, 2002; Zhang et al., 2008). Nitrate uptake rate by plants is higher than other nutrient uptake rates, such as Ca^{2+}, Mg^{2+}, and K^{+} (Seawright et al., 1998). Nitrate uptake kinetic in plants was studied using a ^{15}N tracer model. The results showed that increase in ammonium concentration resulted in higher uptake rate by the plant, and Michaelis-Menten kinetic was the best-fit kinetic model (Inselsbacher et al., 2013; Zhang et al., 2008). However, the preference for ammonium and nitrate depends on their concentrations, growing stages, and plant's genetic factors (Fink and Feller, 1998); thus making it difficult to model nitrogen uptake by plants. Many other empirical models have been established for growth determination of plant by considering the nitrogen content in plant, which is proportional to the growth rate at a certain season (Fink and Feller, 1998). Nitrogen uptake rate was also found to increase over time (age of plant) in a salinized aquaponic system using shrimp (*Litopenaeus vannamei*) and hydroponic tomato (*Lycopersicon esculentum*) (Mariscal-Lagarda and Páez-Osuna, 2014).

Plants growing in aquaponic systems take up nitrate as the main nitrogen source because nitrate concentration in aquaponic systems is higher than ammonium and nitrite concentrations (Hu et al., 2015; Rakocy et al., 2003). Inside the root, water and dissolved minerals are transported via xylem for photosynthesis. The movement of water and minerals is translocated by capillary force in the interconnecting organs and the evaporation from plant's leaves, leading to the suction of water and minerals. The evaporation predominantly occurs at stomata (small pores where oxygen and carbon dioxide normally diffuse between leaves and the atmosphere) (Resh, 2013). Nitrate is assimilated into organic nitrogen by the mediations of many enzymes

such as nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), glutamate synthase (glutamine: 2-oxoglutarate aminotransferase, or GOGAT), glutamate dehydrogenase (GDH), aspartate aminotransferase (Asp-AT), and asparagine synthetase (AS), respectively (Tiaz and Zeiger, 2002). Figure 2.3 shows the pathways and intermediate compounds of nitrogen assimilation by plants. During translocation and assimilation, inorganic nitrogen influx and efflux also takes place between the roots and the recirculating water (Figure 2.5). The reason for nitrate efflux on its biochemical basis has not been fully discovered (Reddy and Ulaganathan, 2015; Segonzac et al., 2007). However, it has been shown that the nitrate efflux is mediated by nitrate transporters such as nitrate transporter 1.5 (NRT1.5) and nitrate excretion transporter 1 (NAXT1) of root plasma membrane (Reddy and Ulaganathan, 2015; Segonzac et al., 2007). The nitrate efflux is induced by stress conditions such as medium acidification, defoliation, mechanical stress, transplant shock, and pathogen attacks (Reddy and Ulaganathan, 2015; Segonzac et al., 2007). This suggests that plants do not fully assimilate nitrogen when nitrate exceeds plant requirement (R.Dave Evans, 2001; Kalcsits and Guy, 2013). Figure 2.5 shows the overall nitrogen transformations with nitrogen species in aquaponic systems.

2.2.8. Nitrous oxide (N_2O) emission

Aquaponic and aquaculture systems can emit nitrous oxide (N_2O), accounting for 1.5-1.9% of nitrogen input in aquaponic systems (Hu et al., 2015; Zou et al., 2016b). Nitrous oxide is a potent greenhouse gas with global warming potential of as high as 310 times of CO_2 over a 100-year lifespan and has also been reported to destroy the stratospheric ozone layer (Chipperfield, 2009; Hu et al., 2013, 2012). It is generated under both anoxic and aerobic conditions. In aquaponic systems, N_2O emission is induced by several conditions such as insufficient denitrifiers population, nitrite inhibition, oxygen inhibition (Hu et al., 2015; Wunderlin et al., 2012), insufficient organic carbon, and low pH (Lu and Chandran, 2010; Zou et al., 2016b). The difference in the oxidation rate between AOB and NOB also leads to accumulation of nitrification intermediates, increasing the generation of N_2O via chemical decomposition and nitrifier denitrification (section 2.2.9). Thus, N_2O emission from aquaponic systems could occur during both ammonia oxidation and denitrification in the grow bed of

media-based aquaponic systems (Zou et al., 2016b) and in the biofilters of floating-raft and NFT systems (Wrage et al., 2001; Zou et al., 2016b).

Different strategies have been adopted to reduce N_2O emission in aquaculture. For example, the addition of starch to stimulate the growth of nitrogen-consuming heterotrophic microorganisms was effective in reducing the daily N_2O emission in intensive aquaculture system (Hu et al., 2014). Aquaponic systems have been recommended to be an alternative method to reduce N_2O emission from aquaculture systems through nitrogen assimilation (Hu et al., 2015). However, studies on N_2O emission from an aquaponic system are still very limited.

2.2.9. Chemical decomposition and nitrifier denitrification

Chemical decomposition is the process in which N_2O and N_2 gas are generated under aerobic condition. After the ammonia oxidization, intermediates are produced before nitrite formation. Hydroxylamine (NH_2OH) and nitroxyl (NOH) are the main intermediates that transform to N_2O via chemical decomposition process. The occurrence of this process is due to high concentration of ammonium and low DO concentration. This condition causes the rate of ammonia oxidization relatively higher compared to that of nitrite oxidation. Hence the intermediates accumulate (Wrage et al, 2001). *Nitrosomonas europaea* was reported as one of the microorganisms producing N_2O through the reduction of nitrite using NH_2OH as an electron donor under limited oxygen or anoxic conditions (Beaumont et al., 2004). Due to the sensitivity of oxygen availability, such microbes produce more N_2O per cell than the autotrophic nitrifying bacteria under anoxic condition (Wrage et al., 2001). Thus, the formation of N_2O during ammonia oxidization at low DO condition takes place in aquaponic systems (Hu et al., 2015).

The other pathway of N_2O and N_2 emissions is via nitrifier denitrification. Nitrifier denitrification is the process in which ammonia oxidation and denitrification take place simultaneously in the absence of nitrite oxidoreductase enzyme when oxygen level is low (Kool et al., 2010; Wrage et al., 2001; Zou et al., 2016b). Also, in nitrifier denitrification, heterotrophic bacteria oxidize ammonium at a slower rate relative to autotroph nitrifiers, causing the accumulation of the intermediates between ammonium and nitrite. Under low DO condition and abundant ammonium in biofilters, nitrifier denitrification coupled with ammonia oxidation also

can significantly contribute to the generation of NO and N_2O in the presence of AOB such as *Nitrosospira* spp. and *Nitrosomonas europia* (Kool et al., 2010; Shaw et al., 2006; Zhu et al., 2013). During heterotrophic nitrification, some bacteria (e.g., *Alcaligenes faecalis* and *Rhodococcus* sp. CPZ24) can perform aerobic denitrification and heterotrophic denitrification simultaneously (Zhao et al., 2012; Chen et al, 2012). For example, *Rhodococcus* sp. CPZ24 was reported to transform 85% of ammonium nitrogen to nitrite and nitrate by 13%, biomass by 24% and gaseous nitrogen by 48% (Chen et al, 2012).

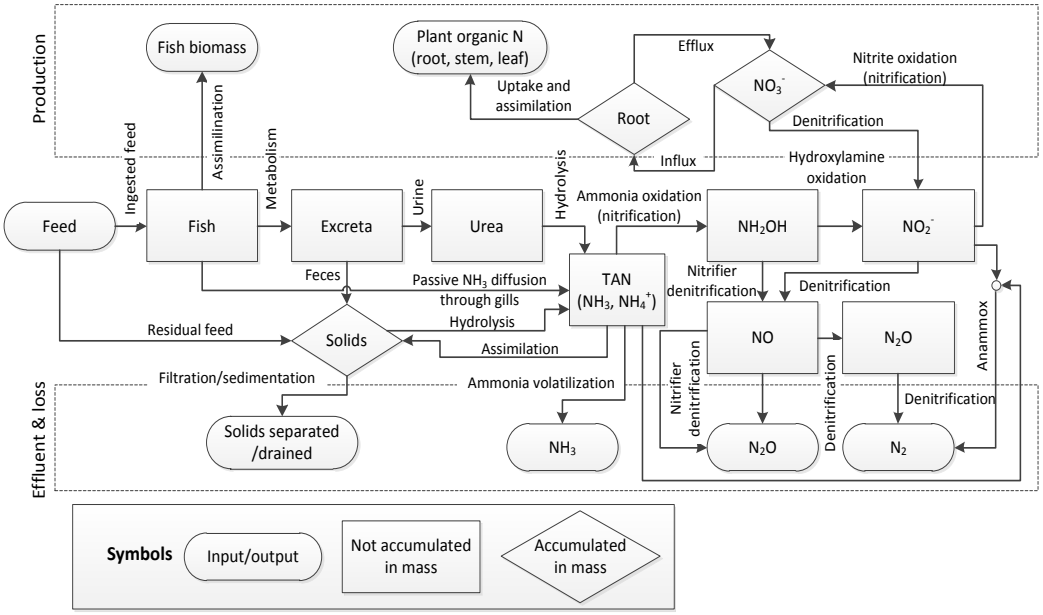

Figure 2.5. Nitrogen transformations and nitrogen species in aquaponic systems (Wongkiew et al., 2017a)

2.2.10. Complete nitrification by a single microorganism

The discovery of a few bacteria responsible for a complete nitrification, called "comammox", was reported in 2015 (Daims et al., 2015; Kuypers et al., 2018; van Kessel et al., 2015). A single nitrification step via comammox requires lower energy for a complete nitrification ($\Delta G^{o'}$ = -349 kJ/mol NH_3) than two processes of AOB ($\Delta G^{o'}$ = -275 kJ/mol NH_3) and NOB ($\Delta G^{o'}$ = -74 kJ/mol NO_2^-) (Daims et al., 2015; van Kessel et al., 2015). This new finding could change the understanding of nitrification in the nitrogen cycle. Using metagenomics and proteomics, comammox was reported to be different from AOB and NOB due to its phylogenetic

difference. Comammox contain the important genes encoding ammonia oxidation (*amo*), hydroxylamine oxidation (*hao*), and nitrite oxidation (*nxr*).

There have been very limited studies on comammox, and it was found to occur in few environments, such as biofilms of an anaerobic compartment of a trickling filter connected to a recirculation aquaculture system (van Kessel et al., 2015), biofilms developing on the walls of a pipe under the flow of hot water (56^{o}C) of a deep oil well (Daims et al., 2015), nitrifying activated sludge, membrane bioreactor of a municipal wastewater treatment system (Daims et al., 2015), ammonia-limited ground water, water supply systems, and soils (Kuypers, 2017). Comammox may prefer substrate limitation environments; however, AOA and AOB were dominant over comammox in forest soils and seawater (Daims et al., 2015; Kuypers, 2017), indicating that other factors, such as the efficiency of organic nitrogen and copper (important in cytochrome c oxidase) uses, affected the abundance of AOB and NOB over comammox. There is no information if comammox or AOB is dominant in aquaponic systems. However, since comammox prefers ammonia-limited environments (Kuypers, 2017), AOB could be possible to be dominant over comammox in aquaponic systems.

Daims et al. (2015) reported that a *Candidus* Nitrospira inopinata (*Ca.* N. inopinata) was phylogenetically closed to Betaproteobacterial *amoA* (Daims et al., 2015; van Kessel et al., 2015). *Ca.* N. inopinata contains the genes of the complete nitrification enzymes, namely nitrite oxidoreductase (NXR), ammonia oxidoreductase (AMO), hydroxylamine dehydrogenase (HAO), cytochrome c_{554} (CycA) and cytochrome c_{m552} (CycB), which are found in AOB for the electron transfer from hydroxylamine to the quinone pool. Similarly, Maartje et al. (2015) found two *comammox Nitrospira* species, namely *Candidus* Nitrospira nitrosa and *Candidus* Nitrospira nitrifican, which had a close AMO genomic region to betaproteobacteria *Nitrosomonas europaea* and gammaproteobacteria *Nitrosococcus oceani* (van Kessel et al., 2015). These candidates also lack enzymes for assimilatory nitrite reduction, which is present in other common *Nitrospira*.

2.2.11. Ammonia volatilization

Ammonia volatilization is considered to be insignificant in well-operated aquaponic systems due to the low concentration of NH_3. However, aeration and mixing increase the

volatilization rate, especially when the pH of recirculating water is above 8.0 (Hargreaves, 1998; Lekang, 2013). Ammonia volatilization rate (g-N/m^2-day) correlates with NH_3 concentration (Zimmo et al., 2004). Since pKa of ammonia is 9.25 at 25^oC, NH_3 concentration is relatively low at neutral pH, which is ideal for aquaponic systems (Bernstein, 2011; Nelson, 2008). At high pH, ammonia volatilization rate is affected by TAN concentration, temperature, and aeration rate in the fish tank (Hu et al., 2012). The ratio of free ammonia-to-TAN in open systems at equilibrium can be approximated by Eq. 2.17 (Babu et al., 2011):

$$\text{Fraction of } NH_3\text{-N/TAN} = 1/(1+10^{(pKa-pH)}) \qquad (2.17)$$

2.3. Factors affecting nitrogen transformations in aquaponic systems

2.3.1. TAN, nitrite and nitrate concentrations

In aquaponic systems, TAN needs to be oxidized to nitrate because nitrate is not toxic to fish even at high concentrations of up to 150-300 mg N/L (Graber and Junge, 2009; Hu et al., 2014). However, TAN and nitrite concentrations have to be maintained at low levels (Buzby and Lin, 2014; Liang and Chien, 2013). For example, TAN and nitrite concentrations of 1.6 to 2.9 mg N/L and 0.4 to 1.1 mg N/L, respectively, were reported in a well-operated aquaponic systems using tilapia and basil while nitrate accumulated at relatively high concentrations of up to 54.7 mg N/L (Rakocy et al., 2003). Other studies showed that the nitrate in aquaponic systems can vary from 10 mg N/L to over 200 mg N/L without stress to tilapia and plants (Lam et al., 2015; Seawright et al., 1998; Sikawa and Yakupitiyage, 2010).

During the start-up period of aquaponic systems or when feeding rate is increased rapidly, sufficient concentration of nitrifying microorganisms in biofilters is needed to steadily oxidize ammonium and nitrite to nitrate. If nitrifiers are not present in biofilters, TAN and nitrite concentrations will increase. High concentrations of TAN and nitrite inhibit nitrifiers and are toxic to fish. Furthermore, nitrite concentration of 27 mg N/L reduced the ammonia oxidation rate by nearly 50% (Nijhof, 1995; Sudarno et al., 2011). Nitrite is potentially toxic to fish because it has an extremely high affinity towards hemoglobin, which reduces the oxygen-carrying ability for cell respiration (Hargreaves, 1998). NH_3 and nitrite concentrations were suggested to be maintained below 0.06 mg N/L and 8.2 mg N/L, respectively (Thomas Popma

and Masser, 1999). The toxicity of TAN is dependent on pH as it governs the distribution of NH_3 (Eq. 2.17), which stresses the osmoregulation of ammonia in the fish (Ip and Chew, 2010).

Insufficient nitrification is a major problem in biofilters, especially during a start-up period. Within two weeks, TAN concentration decreases drastically when the abundance of AOB is completely inoculated in biofilters. However, during this time, the growth rate and the abundance of AOB are relatively higher than those of NOB during the start-up period, leading to the accumulation of nitrite (Delong and Losordo, 2012). Once the AOB and NOB are fully established, the rates of ammonium and nitrite oxidation reach steady state. The supplementation of commercially available nitrifying bacteria together with low recirculation rate during the start-up period can also accelerate the nitrification rate (Delong and Losordo, 2012; Kuhn et al., 2010).

In aquaponic systems, biofilm process (biofilter) is used for nitrification. In engineering design, the removal rate of 0.8 and 1 mg TAN/m^2-d at 20 and 25^{o}C, respectively, were suggested (Lekang, 2013). The removal rate can be used to determine the required surface area of biofilters. The kinetic constants and orders of nitrification reaction are dependent on many factors. Since the aquaponic systems consist of three major entities: fish, plants, and microbial community, more meticulous consideration is needed in adopting the kinetic model.

Michaelis-Menten equation or Monod's equation can be applied for determining nitrification rate of fixed-film biofilters in aquaponic systems. However, zero- or first-order kinetics can be assumed to simplify the calculation (Connolly et al., 2015; Hagopian and Riley, 1998; Müller et al., 2007). Ammonia oxidation rates in both fresh and marine aquaponic biofilters can be expressed as zero-order to first-order reactions (Díaz et al., 2012; Endut et al., 2010). In large-scale hydroponic biofilter (non-recirculating system) treating eutrophic surface water at long retention time, zero-order model was reported for nitrification process of both nitrite and nitrate (Li et al., 2009); thus, the zero-order kinetic model can be applied in non-recirculated aquaponic systems. Moreover, the kinetics of TAN oxidation depended on the hydraulic loading rate (HLR), carbon-to-nitrogen (C:N) ratio and the media characteristics of grow beds in aquaponic systems (Endut et al., 2010). For example, the first-order constant for TAN oxidation was positively correlated with HLR (Endut et al., 2010), while the zero-order

constant for TAN oxidation was negatively correlated with C:N ratio (Michaud et al., 2014). However, the predictability of the models is more accurate when the concentration of DO is sufficient for microbial growth in biofilters.

Nitrate accumulations and nitrate depletion in recirculating water show the imbalance between plant requirement and ammonia generation. Table 2.3 shows the nitrogen concentrations, nitrogen variations, and trends of nitrate in different types of aquaponic system. Nitrate accumulation in previous studies on aquaponic systems (Table 2.3) occurred when nitrate generation rate exceeded the amount of nitrate that plants could utilize (Table 2.3). As a result of the higher nitrate generation than nitrate utilization, nitrate accumulation occurred. This is shown by the trend of nitrate concentration in the recirculating water over time. Conversely, nitrate concentrations decreased over time when nitrate uptake rate by plants was higher than the nitrate generation rate. This decreasing trend of nitrate indicates nitrate depletion in aquaponic systems. The increase in nitrate concentration represents the nitrate accumulation, and the decrease in nitrate concentration represents the nitrate depletion (Table 2.3). Thus, the overall balance of nitrogen in aquaponic systems occurred when the trend of nitrate concentration was constant (stable) over time. However, studies on optimizing nitrogen in aquaponic systems are still in its infancy.

Table 2.3. Nitrogen concentrations, nitrogen variations, and trends of nitrate in different types of aquaponic system (Wongkiew at al., 2017a)

Plant names	Fish names	Types of aquaponics	TAN (mgN/L)	NO_2^- (mgN/L)	NO_3^- (mgN/L)	Trends of NO_3^- over time	References
Tomato (*Lycopersicon esculentum*)	Nile tilapia (*Oreochromis niloticus*)	Floating-raft	5[a] (1-10)[b]	< 0.3	10 (5-18)	Stable NO_3^-	(Hu et al., 2015)
Pak choi (*Brassica campestris L. subsp. Chinesis*)	Nile tilapia (*Oreochromis niloticus*)	Floating-raft	25 (11-33)	< 0.3	23 (16-31)	Stable NO_3^-	(Hu et al., 2015)
Water spinach (*Ipomoea aquatica*)	African catfish (*Clarias gariepinus*)	Floating-raft (with rapid sand filter)	6.97 (1.14-2.68)	0.34 (0.06-0.58)	13.35 (5.4-20.1)	NO_3^- depletion	(Endut et al., 2010)
Pak choi (*Brassica chinensis*)	Common carp (*Cyprinus carpio*)	Media-filled bed	0.7 (0.5-0.9)	< 0.2	37 (16-50)	NO_3^- accumulation	(Zou et al., 2016b)
Basil (*Ocimum basilicum*)	Tilapia (*Oreochromis* sp.)	Floating-raft (batch culture)	2.2 (1.6-2.9)	0.7 (0.4-1.1)	42.2 (26.7-54.7)	NO_3^- accumulation	(Rakocy et al., 2003)
Basil (*Ocimum basilicum*)	Tilapia (*Oreochromis* sp.)	Floating-raft (staggered culture)	1.7 (1.1-2.4)	0.9 (0.5-1.1)	42.9 (30.9-51.8)	NO_3^- accumulation	(Rakocy et al., 2003)
Aubergine (*Solanum melongena*)	Nile tilapia (*Oreochromis niloticus*)	Media-filled bed	N/A (0.03-0.88)	N/A (0.08-0.57)	N/A (1.9-42)	NO_3^- accumulation	(Graber and Junge, 2009)

[a] Average value, [b] Range of water quality variable during trials

Table 2.3 (continued). Nitrogen concentrations, nitrogen variations, and trends of nitrate in different types of aquaponic system (Wongkiew et al., 2017a)

Plant names	Fish names	Types of aquaponics	TAN (mgN/L)	NO_2^- (mgN/L)	NO_3^- (mgN/L)	Trends of NO_3^- over time	References
Tomato and cucumber (*Lycopersicon esculentum* and *Cucumis sativus*)	Eurasian perch (*Perca fluviatilis*)	Media-filled bed	N/A (0.06-0.68)	N/A (0.01-0.18)	N/A (12.1-95)	NO_3^- accumulation	(Graber and Junge, 2009)
Lettuce (*Latuca sativa*)	Trout (*Oncorhynchus mykiss*)	Floating-raft	0.49[a] (0.37-0.59)[b]	N/A	0.35 (0.32-0.39)	NO_3^- depletion	(Buzby and Lin, 2014)
Nasturtium (*Tropaeolum majus*)	Trout (*Oncorhynchus mykiss*)	Floating-raft	0.31 (0.06-0.59)	N/A	0.21 (0.11-0.30)	NO_3^- depletion	(Buzby and Lin, 2014)
Water spinach (*Ipomoea aquatica*)	Marble goby (*Oxyeleotris marmorata*)	Floating-raft (with rapid sand filter)	18 (14-25)	0.6 (0.5-0.8)	30 (15-43)	NO_3^- depletion	(Lam et al., 2015)
Water spinach (*Ipomoea aquatica*)	Tilapia (*Oreochromis* sp.)	Floating-raft	2.8 (0.1-5.2)	0.6 (0.1-0.9)	26.8 (0.1-40.0)	NO_3^- accumulation	(Liang and Chien, 2013)
No plants	Chinese catfish (*Clarias fuscus*)	Intensive aquaculture system	0.14 (0.10-0.17)	0.04 (0.02-0.07)	137 (121-158)	NO_3^- accumulation	(Hu et al., 2013)

[a] Average value, [b] Range of water quality variable during trials

2.3.2. pH

In aquaponic systems, pH is the main factor that controls fish metabolism and microbial activities and affects the availability of nitrogen to plants (Kuhn et al., 2010; Tiaz and Zeiger, 2002; Zou et al., 2016b). The biological oxidations of ammonium and nitrite, and the activity of nitrifiers decreases when the pH is below 6.4 or above 9.0 (Ruiz et al., 2003). However, nitrogen use efficiency (NUE, percent nitrogen input transformed to nitrogen in fish and vegetable) in a media-based aquaponic system was found at maximum of 50.9% when the pH was maintained at 6.4 and NUE dropped to 47.3% and 44.7% when pH was increased to 7.4 and 8.0, respectively (Zou et al., 2016b). This could be because pH governs the solubility of other micro-nutrients such as calcium, phosphorus, potassium, magnesium, etc., which affects the bioavailability of nutrients for plant uptake (Resh, 2013). In addition, N_2O emission in aquaponic systems at low pH conditions was reported to be higher than that in neutral pH conditions due to the inhibition of functional genes such as genes encoding ammonia monooxygenase, nitrite reductase and nitric oxide reductase (Zou et al., 2016b).

pH in an aqueous phase is buffered by alkalinity. Alkalinity of 100 to 150 mg/L as $CaCO_3$ is recommended for aquaponic systems (Delong and Losordo, 2012; Nelson, 2008; Timmons et al., 2002). pH can be periodically adjusted by using potassium hydroxide (KOH) and calcium hydroxide ($Ca(OH)_2$) (Rakocy et al., 2003). KOH and $Ca(OH)_2$ also supply the essential nutrients to plants. In addition, alkalinity can be increased with a slight increase of pH by adding weak bases such as calcium bicarbonate ($Ca(HCO_3)_2$).

2.3.3. Dissolved oxygen (DO)

DO decreases in biofilters, around the root zone of plants in aquaponic systems, biofilters, and fish tanks due to the activities of aerobic microorganisms (e.g., nitrifiers and heterotrophs) and fish (Hagopian and Riley, 1998). Effect of DO on nitrogen transformations has not been well studied in aquaponic systems. Studies using synthetic wastewater (225-450 mg/L of TAN) showed that the activity of AOB reduced when DO level was below 4.0 mg/L, while the activity of NOB decreased at DO below 2.0 mg/L (Kim et al., 2005). DO concentration of above 1.7 mg/L was recommended in biofilters to maintain the activity of nitrifiers (Ruiz et al.,

2003). However, the recommended DO in fish tanks and the inlet of grow beds is 5.0 to 6.0 mg/L to avoid the stress to fish and plants (Bernstein, 2011; Rakocy, 2007). In particular, root rot symptoms may occur when DO is insufficient at high water temperatures (Rakocy, 2007). DO in an aqueous phase is also affected by liquid flow pattern and temperature. Aeration systems can be installed at the inlet of grow bed to prevent anoxic zones (Timmons et al., 2002).

2.3.4. Hydraulic loading rate (HLR)

Hydraulic loading rate (HLR) is defined as liquid flow rate per unit surface area of grow bed (m/day or m^3/m^2-day). The optimum contact time of nutrients and microbes present in the aqueous phase with plant roots is governed by HLR (Li et al., 2009). The highest NUE of plants in a media-based aquaponic system was reported at the HLR of 1.28 m/day (Endut et al., 2010). Fish biomass yields were not significantly different at different HLRs (Endut et al., 2010). Low HLR leads to oxygen deficiency for fish, plants, and microbes in the aqueous phase. In contrast, high HLR reduces the contact time between the recirculating water and the plant roots, and high HLR also washes out the microbes and the sediment attached on the plant roots and biofilter media (Endut et al., 2010; Prehn et al., 2012).

2.3.5. Carbon to nitrogen (C:N) ratio

Inorganic nitrogen in aquaponic systems can be converted into plants as long as the nitrogen is not transformed to microbial biomass or loss as nitrogen gas. However, high C:N ratio reduces the abundance of nitrifiers and the nitrification efficiency because the growth rate of nitrifiers is lower than that of heterotrophs (Ebeling et al., 2006; Michaud et al., 2014). At high C:N ratio, heterotrophs assimilate ammonium and nitrate in the presence of organic carbon for cell growth, and turn into sludge, which reduces the nitrogen availability for plant uptake. The high growth rate also results in dominance of heterotrophs over autotrophs at high C:N ratio (Ebeling et al., 2006; Michaud et al., 2014). High concentration of heterotrophs in biofilters drastically lowers the DO and promotes anoxic condition, which results in the emissions of N_2O and N_2. Thus, C:N ratio affects nitrification efficiency, nitrogen emissions, and nitrogen availability for plant uptake.

Various factors affecting nitrogen transformations, operating parameters, and their relationships on nitrogen transformations in aquaponic systems are presented in Figure 2.6.

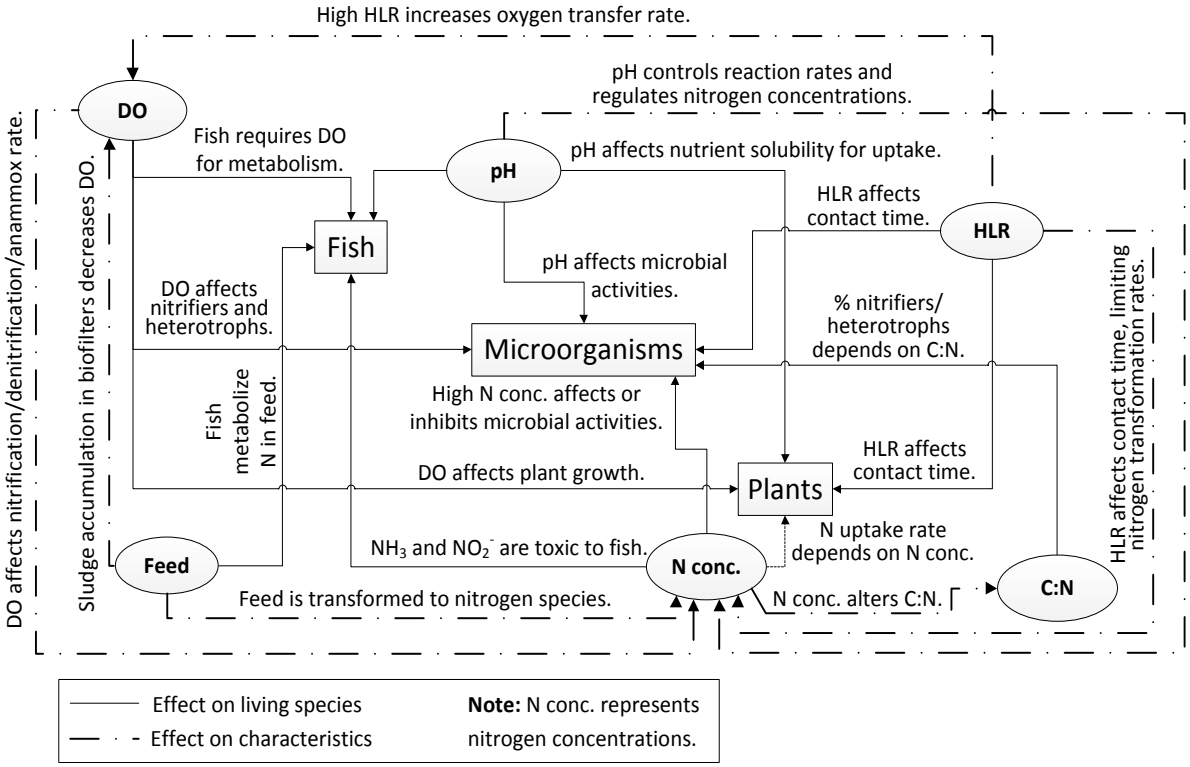

Figure 2.6. Effects of DO, pH, HLR, C:N ratio, nitrogen concentrations, and feed on fish, microorganisms, and plants in aquaponic systems (Wongkiew et al., 2017a)

2.4. Significance of stable isotope in ecology

2.4.1. Nitrogen isotope in ecology

According to Ernest Rutherford's atomic model, one atom consists of protons, electrons, and neutrons. Protons and neutrons cluster together at a center, called nucleus, while electrons move around the nucleus. Proton has a positive charge while a neutron is neutral. Mass of one atom is dependent on the numbers of protons and neutrons. Thus, different atoms can have different masses if they have different numbers of neutrons and protons (Fry, 2006).

Any elements that have two (or more than two) different numbers of neutrons are called isotope. For example, nitrogen (N) in nature has of two stable isotopes. Nitrogen consists of

seven protons and seven electrons, but nitrogen can have seven or eight neutrons in nature (Fry, 2006). Because a mass of an element includes protons and neutrons, two nitrogen isotopes have different masses: 14 (7+7) and 15 (7+8) mass numbers. Thus, ^{14}N and ^{15}N are two forms of nitrogen stable isotopes. The superscripts 14 and 15 mean mass numbers; hence, an atom of ^{15}N is heavier than an atom of ^{14}N. In this context, ^{15}N can be called heavy isotope, and ^{14}N can be called light isotope (Robinson, 2001). ^{14}N and ^{15}N are stable isotopes; they are non-radioactive, and their masses are constant.

Compounds and elements have their isotopes. For examples, two nitrogen isotopes can be found in nitrate as $^{15}N^{16}O_3^-$ (mass number = 63) and $^{14}N^{16}O_3^-$ (mass number = 62). Nitrogen gas has two isotopes namely $^{15}N_2$ (mass number = 30) and $^{14}N_2$ (mass number = 28). In nature, the percentage of light isotope is much higher than heavy isotope. For instance, dinitrogen (N_2) gas in the air contains 99.63370% of light isotope (^{14}N) and 0.36630% of heavy isotope (^{15}N) (Fry, 2006).

2.4.2. Notations and isotope mass balance calculations

When applying isotope to ecological studies, a ratio of heavy isotope to light isotope is normally used to represent an isotope value. There are many notations of isotope value, such as isotopic composition (δ), isotope ratio (R), and fractional abundance (Hayes, 2004). Among those, the notation δ (delta) is normally used to read and compared with other δ values because natural abundance δ varied suitably in this scale (per mil, ‰), and δ is based on the isotope ratios of samples and a standard, which allow the isotope ratios to be easily reported internationally. However, calculations based on δ can lead to misleading in isotope mass balance (see section 2.4.3) when isotope labeling (e.g., enriched ^{15}N) is studied. Atom percent or fractional abundance is always correct and recommended to use instead of δ. Using δ in natural abundance isotope study is acceptable and do not lead to misinterpretation (Fry, 2006; Hayes, 2004). Isotopic composition (δ), isotope ratio (R), fractional abundance, and atom percent of nitrogen (^{15}N) can be expressed as followed (Fry, 2006; Hayes, 2004):

$$\text{Atom percent} = [^{15}N/(^{15}N+^{14}N)] \times 100 \qquad (2.18)$$

$$\text{Fractional abundance of } ^{15}N = {}^{15}N/(^{15}N+^{14}N) \qquad (2.19)$$

$$\text{Nitrogen isotope ratio} = R = {}^{15}N/{}^{14}N \qquad (2.20)$$

$$\text{Nitrogen isotopic composition} = \delta^{15}N = [(R_{sample}/R_{satandard}) - 1] \times 1000 \qquad (2.21)$$

Where ^{15}N and ^{14}N are the percentages of heavy and light nitrogen in a compound, respectively. For example, dinitrogen (N_2) gas in the air contains 99.63370% light isotope (^{14}N) and 0.36630% heavy isotope (^{15}N); thus, nitrogen isotope ratio (R) is 0.0036765 (= 0.36630/99.63370). For isotope measurement, the isotope ratio of air has been used as international reference standard. Thus, $R_{standard}$ for nitrogen isotope analysis is 0.0036765 (air) (Fry, 2006).

2.4.3. Mass balance and isotope mass balance

Nitrogen mass balance techniques have been employed to evaluate the nitrogen budgets in natural and engineered ecosystems, including aquaponic systems. Mass is conserved in chemical and physical processes. The mass balance approach provides information on the yield of products relative to the input. Assuming no leak in a system boundary, a mass balance equation for a system (e.g., a bioreactor) can be written as (Doran, 1995):

$$[\text{mass in}] - [\text{mass out}] + [\text{mass generated}] - [\text{mass consumed}] = [\text{mass accumulated}] \qquad (2.22)$$

Considering Eq. 2.22, mass coming in a system boundary can accumulate in the systems. [mass in] also can be consumed by some living species and transformed into other mass products; the rest can go out of the system boundary. In other words, mass coming into a system will eventually change to other products and loss. Mass balance can be shown by Eq. 2.23 because mass is conservative. In addition, total mass equals the summation of single products (e.g., mass no.1, mass no.2, etc.) (Eq. 2.24) (Hayes, 2004).

$$[\text{mass}]_{in} = [\text{mass}]_{product} + [\text{mass}]_{loss} \qquad (2.23)$$

$$\Sigma\,[\text{mass}] = [\text{mass}]_1 + [\text{mass}]_2 + \ldots \qquad (2.24)$$

Nitrogen isotopic mass balance is a modification of nitrogen mass balance. Since total isotopic abundance is conserved in one system, the mass of heavy isotope in the system is conservative. Because the fractional abundance or atom percent of ^{15}N represents a fraction of

^{15}N in a mass, isotope mass balance equations can be written based on mass balance equations (Eqs. 2.23 and 2.45) and shown as Eqs. 2.25 and 2.26 (Hayes, 2004).

$$F_{in} \times [mass]_{in} = F_{product} \times [mass]_{product} + F_{loss} \times [mass]_{loss} \qquad (2.25)$$

$$F_{mixed} \times \Sigma [mass] = F_1 \times [mass]_1 + F_2 \times [mass]_2 + \ldots \quad (2.26)$$

F can represent either atom percent or fractional abundance of ^{15}N.

To simplify the isotope mass balance equation for natural abundance isotope calculation, equations 2.25 and 2.26 can be expressed by replacing the Fs with δ values, as shown in Eqs. 2.27 and 2.28. Errors from these equations are acceptable when applying with natural abundance isotope variations (section 2.4.5). Eqs. 2.27 and 2.28 should be avoided when artificial labeled (section 2.4.5) mass is studied (Hayes, 2004).

$$\delta_{in} \times [mass]_{in} = \delta_{product} \times [mass]_{product} + \delta_{loss} \times [mass]_{loss} \qquad (2.27)$$

$$\delta_{mixed} \times \Sigma [mass] = \delta_1 \times [mass]_1 + \delta_2 \times [mass]_2 + \ldots \quad (2.28)$$

2.4.4. Isotope effect and fractionation

An isotope effect is a physical phenomenon (e.g., an atomic-level vibration of a molecule) that causes some slight differences in atomic physical properties of a molecule (e.g., molecular bond, kinetic energy) during a reaction (Hayes, 2004). An isotope effect leads to a fractionation (a change in atom percent, fractional abundance, R, and $\delta^{15}N$ value), which is observable, between a substrate and a product (Hayes, 2004). A magnitude of an isotope effect is proportional to an observed fractionation, which is measurable during a reaction (Hayes, 2004). There are two types of reactions that can cause isotope fractionations: kinetic-based reaction and equilibrium-based reaction. Thus, isotope fractionations can be caused by kinetic isotope effects (KIE) and equilibrium isotope effects (EIE) (Fry, 2006; Hayes, 2004). Using carbon as an example, kinetic isotope effect can be described by Eq. 2.29, which refers to acetyl coenzymeA formation in a biochemical process (Hayes, 2004). Pyruvate is transformed into acetyl conenzymeA and carbon dioxide by pyruvate dehydrogenase enzyme. Here, carbon has two stable isotopes: mass = 12 and 13.

Kinetic: $CH_3COCOOH \rightarrow CH_3COS(CoA) + CO_2$ (2.29)

KIE can be described based on the fact that light isotope molecules react faster than heavy isotope molecules (Fry, 2006). Thus, the products will have a higher amount of light isotopes than the substrate (pyruvate). Assuming k refers to a kinetic rate, the rates for molecules with light and heavy isotopes can be written as ^{12}k and ^{13}k, respectively. Since light isotopes react faster than heavy isotopes, the ratio of ^{12}k to ^{13}k should be higher than 1.0. For example, the $^{12}k/^{13}k$ value of the acetyl coenzymeA relative to the pyruvate is 1.0232 (Hayes, 2004). This value is called fractionation factor (α), which is analogous to rate constant ($\alpha = {}^{12}k/{}^{13}k$) and the isotope ratio of the product to the isotope ratio of the substrate ($\alpha = R_{product}/R_{substrate}$) (Eq. 2.30). The α value is interchangeable with an isotope effect value (ε). The relationship between ε and α can be shown in Eq. 2.31 (Robinson, 2001).

$$\alpha = R_{product}/R_{substrate} \quad (2.30)$$

$$\varepsilon = (\alpha - 1) \times 1000 \quad (2.31)$$

EIE, in contrast, is based on the rule that heavy isotopes concentrate in which it is most strongly bonded (Bender et al., 1997; Fry, 2006; Wolfsberg, 1972). Using carbon as an example, EIE can be described by Eq. 2.32, which refers to the equilibrium of carbon dioxide and bicarbonate (dissolved ions). At an equilibrium state, heavy isotopes will concentrate and bind to bicarbonate molecules in which their molecular bonds are stronger than carbon dioxide. In EIE, fractionation factor will be similar to equilibrium constant ($\alpha = R_{HCO3-}/R_{CO2}$), not rate constant. For example, $\alpha_{HCO3-/CO2} = 1.0068$ at 30 ^{o}C.

Equilibrium: $^{13}CO_2\,(g) + H^{12}CO_3^-\,(aq) \leftrightarrow {}^{12}CO_2\,(g) + H^{13}CO_3^-\,(aq)$ (2.32)

Mostly in biological and biochemical processes, KIE is more likely occurs than EIE (Robinson, 2001). KIE causes two different $\delta^{15}N$ values between a substrate and a product; thus, reflecting an isotope fractionation. The magnitude of isotope fractionation, fractionation value (ε), depends on the difference between a quantity of heavy isotopes and a quantity of light isotopes that turn into a product. An incomplete fractionation can occur when a substrate does not completely transform to a product. In this case, the difference between $\delta^{15}N$ values of a

substrate and a product will be variable and depend on the fraction of the substrate that is used in a reaction (f). The difference between $\delta^{15}N$ values of the substrate (δ_0) and the product (δ_s) can be mathematically shown by Rayleigh equation (Eq.2.33) (Robinson, 2001). Since ln1 equals 0, there will be no fractionation if a substrate does not transform to a product. The product becomes ^{15}N depleted relative to the substrate if fractionation occurs. The ε value is constant in a certain condition.

$$\delta_s - \delta_0 = \varepsilon \ln(1-f) \qquad (2.33)$$

In fact, Rayleigh equation only describes fractionations in unidirectional reactions in closed systems (Robinson, 2001). However, the equation can be used in open systems with irreversible reactions (e.g., aquaponic systems) by assuming that a substrate is continuously and sufficiently supplied to the systems (Hayes, 2004; Robinson, 2001). As the results, f will be constant at a steady state (Mariotti et al., 1982). Fractionation value (ε) in the open systems can be calculated by Eq. 2.34, which is derived by combining Eqs. 2.21, 2.30, and 2.31. In many cases, if natural abundance $\delta^{15}N$ values of products are low (< 10 ‰), ε can be simplified to Eq. 2.35.

$$\varepsilon = 1000 \times (\alpha-1) = 1000 \times \{(\delta_0 - \delta_s)/[1+(\delta_s/1000)]\} \qquad (2.34)$$

$$\varepsilon = \delta_0 - \delta_s \qquad (2.35)$$

In the nitrogen cycle, ε varies depending on biological processes and external condition such as temperature. Table 2.4 shows the variations of ε values in different nitrogen cycle processes. Those values can be used to evaluate nitrogen cycle processes or predict a $\delta^{15}N$ value of an input or an output when either one is unknown. These values in Table 2.4 are also useful to solve isotopic mass balance calculation.

Table 2.4. Fractionation values in nitrogen cycle processes

Process	Fractionation value (ε) (‰)
N_2 fixation via nitrogenase	0 – 6
NH_3 volatilization	40 – 60
N_2O and NO production during nitrification	35 – 60
N_2O and NO production during denitrification	28 – 33
NO_3^- assimilation to organic nitrogen by plants	0 – 19
NH_4^+ assimilation to organic nitrogen by plants	9 – 18
NO_3^- or organic nitrogen assimilation by microbes	13
NH_4^+ assimilation by microbes	14 – 20
NH_4^+ production during ammonification	0 – 5
NO_3^- production during nitrification	15 – 35
Organic nitrogen assimilation by animals (deamination and transamination)	1 – 6

Source: Robinson (2001)

2.4.5. Natural abundance vs. labeling nitrogen isotope study

Natural abundance stable nitrogen isotopic compositions of nitrogen compounds have been widely employed to identify the microbial processes involved in nitrogen transformations in ecological and biogeochemical studies (e.g., Robinson, 2001; Onodera et al., 2014; Ryabenko, 2013). The $^{15}N/^{14}N$ ratios or $\delta^{15}N$ values in different compounds are not identical due to the isotopic fractionation caused by different physical, chemical, and biochemical reactions. For example, nitrogen metabolism in fish results in waste produced depleted in ^{15}N relative to fish feed. By mass balance, other metabolic products such as fish muscle tissue and feces become enriched in ^{15}N. Moreover, the total nitrogen isotopic ratio in a system is largely conserved, such that if one product is enriched in ^{15}N (increase in $\delta^{15}N$ value), another product must become depleted in ^{15}N, and vice versa. This approach does not require the addition of an enriched ^{15}N source, which can permanently alter the $\delta^{15}N$ values of the system. In aquaponic systems, natural abundance nitrogen isotopic fractionation associated with nitrogen transformations is typically

large enough to easily identify the mechanisms of nitrogen transformations, despite the slow metabolisms of fish, plants, and microorganisms. However, isotope effects must be considered in the natural abundance isotope mass balance due to the fractionations of mass input and mass outputs.

Labeling isotope study, sometimes called isotope addition or enriched isotope, is the method that adds a high amount of external heavy isotopes to overwhelm natural abundance isotopic composition so that isotope effects can be disregarded (Fry, 2006). Normally, an external tracer that contains more than 10% atom percent heavy isotope (e.g., $(^{15}NH_4)_2SO_4$, $K^{15}NO_3$) is added to a system of interest (i.e., $^{15}NO_3^-$ is added to track denitrification pathway) (Fry, 2006). This isotope addition can increase the $\delta^{15}N$ value in a system up to above 1000 ‰. In many cases, up to 90-99 at% (atom percent) of heavy isotopes were added to a system to avoid an increase in substrate concentration, which might affect the turnover rates of the whole biological reactions. In labeling isotope study, $\delta^{15}N$ values of a substrate and products will be expected to be identical if the products of interest are produced from only one labeled element in a substrate. In this way, researchers can evaluate where the labeled substrate goes. The isotope labeling can easily and quickly tell about the mass contributions of input to the products. However, there are two disadvantages of labeling isotope study. Firstly, labeling isotope will lead to misinterpretation if experimental time is too short to allow all outputs overwhelmed by the enriched isotope. If time is too short, the interpretation will reflect only isotope mixing (Fry, 2006). Models of the slow turnover processes should be considered together with mixing when experiments are too short. Secondly, the enriched isotope can permanently alter the $\delta^{15}N$ values of the system. Thus, enriched isotopes in closed systems have to be completely washed prior to additional experiments. Moreover, open systems may need a long time to remove the enriched isotope. Sometimes it is also costly to add a high amount of an enriched isotope when a natural abundance concentration is high.

Overall, isotope labeling is a powerful technique to determine the rates and source contributions at a specific time. Isotope labeling can be used together with natural abundance technique that applied widely in ecosystems. Being used together, isotope labeling and natural abundance can support each other and complement identifying nitrogen transformations, source contributions, and biochemical processes in an ecosystem.

2.5. Advanced molecular techniques for microbial community

Linking microbial community to nitrogen transformations is essential to improve NUE and increase the nitrification efficiency in aquaponic systems. Quantitative polymerase chain reaction (qPCR) and next-generation sequencing (NGS) have been widely used to examine microbial community in aquaponic systems as briefly discussed in Introduction section. Since qPCR and NGS were used in this study, understanding of the two molecular techniques are described in sections 2.5.1 and 2.5.2.

2.5.1. Polymerase chain reaction (PCR) and amplification of DNA

Polymerase chain reaction (PCR) is a method of amplifying specific deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) sequences (Madigan et al., 2014). PCR can quickly and effectively produce billion of copies from a single strand DNA or RNA segment. The process that produces copy segments of DNA or RNA via PCR is called PCR amplification (Madigan et al., 2014). During the DNA amplification, PCR needs four components including 1) a target DNA molecule, 2) a pair of DNA primers to initiate DNA synthesis, 3) heat-resistant DNA polymerase to copy DNA molecules, and 4) four types of deoxyribonucleoside triphosphate, a component containing four bases (A, T, C and G). In PCR, flanking sequence on the nucleotide needs to be known because the flanking sequence is needed to produce DNA primers for replication (Madigan et al., 2014). Three steps for PCR include: 1) denaturing of double helix by heating, 2) annealing of DNA primers, and 3) replication of DNA with heat-resistant DNA polymerase. These three steps are temperature-dependent. The three steps are repeatable by changing the temperature of the solution, and the steps are controlled by an automated PCR machine, called a thermocycler. After one cycle of PCR, the number of DNA molecules will be doubled; and the cycle is repeated until the number of DNA copies is satisfied (usually 20-30 cycles) in few hours (Madigan et al., 2014).

The initial or final amount of target DNA or RNA in a sample can be quantified by applying PCR process. This technique is called quantitative PCR (qPCR) (Madigan et al., 2014). This qPCR uses fluorescence probe and the fluorescence light to monitor and quantify the amount of DNA or RNA during the PCR cycles. The amount of target DNA can be determined

by the rate of fluorescence light, which increases during the PCR reaction. In addition, qPCR can be monitored continuously and in real-time. This means the process for qPCR analyses takes only a few hours, and gel electrophoresis or another additional method that takes overnight is not necessary (Madigan et al., 2014). In molecular microbiology, qPCR has many advantages such as high sensitivity, high specificity to a target, high efficiency, and high simplicity relative to other molecular techniques.

2.5.2. Next-generation and Ion Torrent sequencing

Sequencing is the molecular approach to determine the order of aligned nucleotides in DNA or RNA (Pevsner, 2015). New sequencing technologies that have emerged in recent years are called next-generation sequencing (NGS). NGS is computer based-method that uses automated functions of a computer to detect signals of the sequences of nucleotides and automatically generate nucleotide datasets as files in computer formats, such as FASTA and FASTQ. Since DNA sequencing technology is advancing rapidly, there are many sequencing methods that have been widely employed, such as Sanger dideoxy method, 454 Pyrosequencing, Illumina, SOLiD, and Ion Torrent. NGS can save cost and time for determining a sequence of nucleotides. Here only Ion Torrent is reviewed because only Ion Torrent has been employed in this study.

In Ion Torrent sequencing, before sequencing, double strands of DNA are broken apart into single strands DNA. The single strands then are broken apart into fragments, called reads. Ion Torrent reads nucleotides in DNA fractions by measuring pH during the reactions of DNA polymerase (Pevsner, 2015). Firstly, DNA polymerase is needed to start the reaction. When DNA polymerase incorporates a nucleotide in a strand of DNA fractions, a hydrogen ion is released as a nucleotide signal (Pevsner, 2015). The hydrogen ion released results in a pH value. The sequencing machine detects the voltage of pH, distinguishes the voltage, and translates it into one of four bases (A, T, C, or G) (Pevsner, 2015). This method directly detects the chemical reaction in a semiconductor chip using an ion sensor. Finally, the machine generates an output, a computer format FASTQ file. FASTQ contains sequences of bases and other bioinformatics information. The FASTQ file can be used in a computational analysis (bioinformatics) to create the microbial community. The computational analysis is the workflow of the sequence analysis

such as quality assessment (trimming, filtering), alignment to reference genome, identification, annotation, and taxonomy classification (Pevsner, 2015).

One pitfall of NGS is that software for computational analyses is based on different algorithms and assumptions, such as sequencing quality control, statistic values, and computational models (Pevsner, 2015). For example, one input (FASTQ) can result in different microbial communities if the assumptions for alignment and quality control are different. Thus, it is critical to have a developed a standard operating procedure and a workflow for each type of work.

CHAPTER 3

MATERIALS AND METHODS

3.1. Aquaponic system setup and operation

Six floating raft aquaponic systems were operated in parallel in a greenhouse at the University of Hawaii at Manoa for nearly four years. Each aquaponic system consisted of a fish tank (volume = 335 L), 2-stage biofilter (upflow and down flow with partial aeration, volume = 15 L), and a hydroponic bed (grow bed) (volume = 300 L, area = 1.5 m^2) (Figure 3.1). Water at the mid-depth of the fish tank was pumped to the 2-stage biofilter (a fixed-bed upflow biofilter followed by a downflow trickling biofilter). The first biofilter (upflow) was designed to contain Kaldnes plastic media for attached-growth microbes and capture solids from the fish tank. The second biofilter was designed to perform partial aeration to increase DO concentration for plant roots in grow beds. The biofilter was the only component where an accumulation of solids (sediment) took place during the operation. Kaldnes filter media (surface area $\geq$ 800 m^2/m^3) were used in the upflow biofilter to promote nitrification. The surface area of the filter media was designed to achieve ammonia removal rate at a maximum constant feeding rate of 50 g feed/day. In this design, the production rate of total ammonia nitrogen (TAN) was approximated based on protein content in fish feed (40%) and feeding rate (50 g feed/day) (Hu et al., 2012; Wongkiew et al., 2017a). The required total surface area of the filter media was calculated based on TAN conversion rate of 1.0 g/m^2-day (Timmons et al., 2002). The aquaculture effluent from the downflow tricking biofilter enters the hydroponic bed and then recirculates to the fish tank. The fish tank was constantly aerated with an aquarium pump, and fish were fed daily with fish feed.

The aquaponic systems were operated with four plant species, namely pak choi (*Brassica rapa* L. *Chinensis*, harvesting cycle of 37 days), lettuce (*Lactuca sativa longifolia* cv. *Jericho*, harvesting cycle of 32 days), chive (*Allium schoenoprasum L.*, harvesting cycle of 70 days), and tomato (*Lycopersicum esculentum*, harvesting cycle of 90 days). These species were selected in this study due to their popularity as vegetables and better growth in soilless systems in tropical regions (Kratky, 2010). Each grow bed contained a single raft (24 plants per raft for pak choi, lettuce, and chive, and 6 and 12 plants per raft for tomato). The vegetable seeds were germinated for 2 weeks before being transplanted into the grow beds.

Tilapia (*Oreochromis* spp.) was used as the growing fish due to toleration to pH, temperature, and DO and TAN concentrations. Tilapia also is a very common protein source in many developing countries, and it is a warm-water species that grow well in recirculating tanks (T. Popma and Masser, 1999). Tilapia in each tank at an average stocking density of 17.8 ± 8.0 kg/m^3 were fed once a day (35 g/fish tank-day, 0.60 ± 0.27 % feed/fish weight) with commercial fish feed, classic trout, 3.5 mm diameter (Skretting, UT, USA). However, in some experiments (sections 3.2.2 and 3.2.4), feeding rates were varied from 15-50 g/day (0.26-0.85 % feed/fish weight) to study the effect of feeding rates on nitrogen transformations. Feeding rates were kept constant at a harvesting cycle.

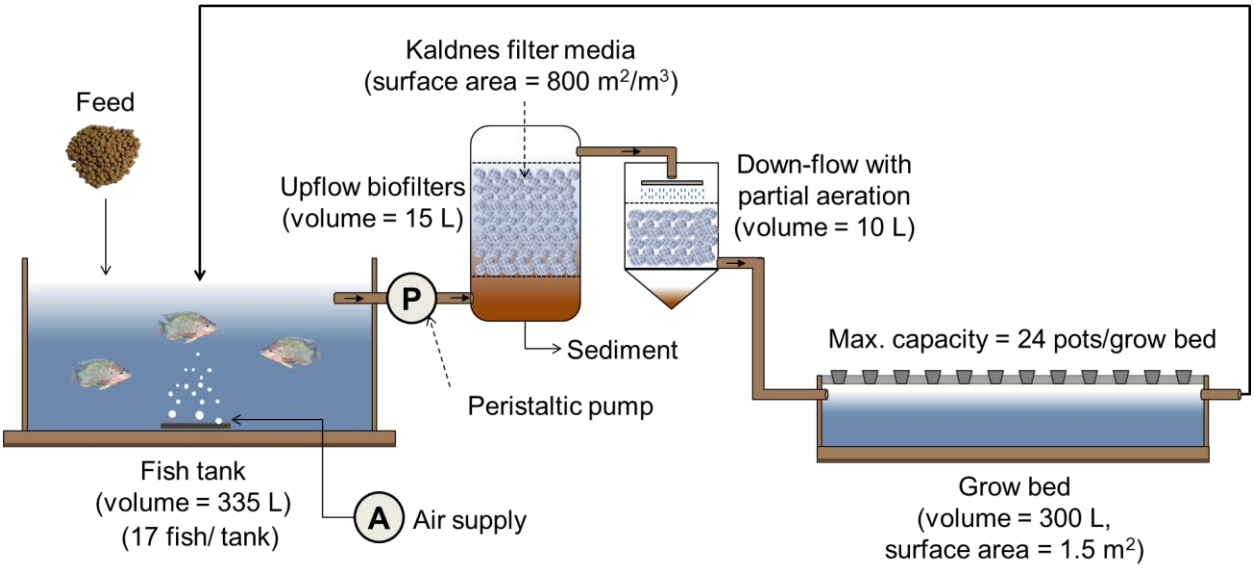

Figure 3.1. Schematics of a floating-raft aquaponic system

Water in the fish tanks was continuously mixed by aeration using fine diffusers, and the HLR was kept constant in each experiment. The aquaponic systems were operated at an HLR of 1.5 m^3/m^2-day and a DO concentration above 6.0 mg/L. However, in some experiments (e.g., Figures 3.2 and 3.3), HLRs and DO concentrations were varied to study the effects of HLRs and DO concentrations on nitrogen transformations (section 3.2.1). The DO in the fish tanks were maintained by constant aeration. The water temperature remained between 25 to 30 ^{o}C (measured daily at noon, see sections 3.3 for sampling and 3.4 for analytical methods) throughout the operation of aquaponic systems and was not controlled. This range of temperature

is considered within the comfort range for the growth of tilapia (Thomas Popma and Masser, 1999). The pH measurement and adjustment (pH ~ 6.8-7.2) were made manually once a day by adding a 1:2 mixture of KOH:$Ca(OH)_2$ by weight (Rakocy, 2007). The volume of recirculating water in the aquaponic systems was held constant by adding tap water to each fish tank daily to compensate the water loss by evaporation and evapotranspiration. Organic nitrogen, TAN, nitrite and nitrate concentrations in the tap water were below detectable levels, and no additional nutrients were added to the system. The whole accumulated solids in the first biofilter was withdrawn at the end of each harvesting cycle. Total biomass of the withdrawn solid was represented by the term "sediment" in this study. Chelated iron DTPA (diethylenetriamine pentaacetic acid) solution was added to each aquaponic systems at the beginning of each planting to raise iron concentration to about 2.5 mg Fe/L (Rakocy et al., 2003). The plants did not show a symptom of a nutrient deficiency, and there was no fish death during all experiments.

3.2. Experimental designs

In this section, experimental design will be described based on four specific objectives, as previously shown in the introduction. Each specific objective includes more than one experimental design. To describe each experimental design, the author used the specific objectives to represent the titles of experimental designs.

3.2.1. Quantify the impact of physical and chemical variables that regulate nitrogen transformations in aquaponic systems

The author hypothesized that HLR was a physical parameter, and DO and pH were the chemical parameters that impact nitrogen transformations in aquaponic systems. Aquaponic systems were operated at varying HLRs (e.g., 0.10, 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 m^3/m^2-day), three pH levels (~7.0, ~6.0, and ~5.2), and two DO concentrations (low DO versus high DO levels, see values in Figure 3.2). Low DO conditions are defined as the minimum DO level in which fish consumed feed without stress (T. Popma and Masser, 1999), and high DO conditions are defined as the DO levels in which a fish tank was supplied with a maximum aeration rate of an air pump used in this study (16 L/ minute). At a harvesting cycle, two conditions were compared, and the aquaponic systems were run in triplicate. To study the effects of HLR, DO, and pH on nitrogen transformations, the author selected plant species based on their capability

and suitability to grow at different conditions and experimental timeframe (see details in each bullet below). Figure 3.2 shows the diagram of the experimental design for the objective 1.

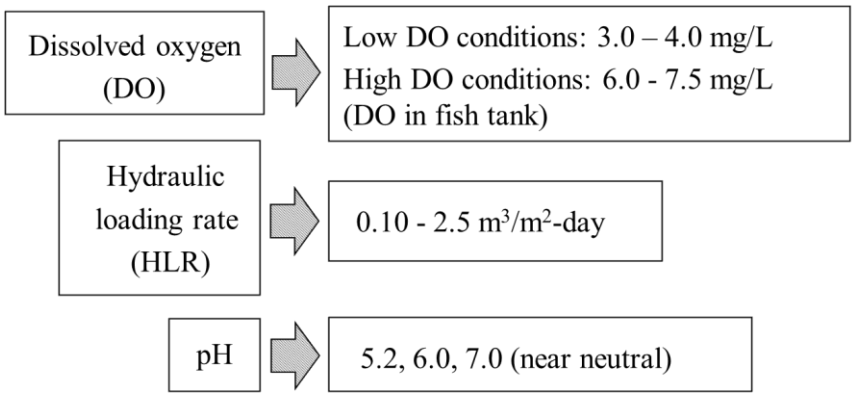

Figure 3.2. Experimental diagram for evaluating effects of DO, HLR, and pH on nitrogen transformations in aquaponic systems

- **Effect of DO on nitrogen transformations:** Nitrogen transformations in aquaponic systems under low DO levels (3.0-4.0 mg/L) were compared with high DO levels (DO = 5.9-7.4 mg/L) (Figure 3.2). Pak choi-based aquaponic systems were used to study the effect of DO on nitrogen transformations. Pak choi was a leafy vegetable that can grow well in aquaponic systems at low DO levels and warm temperature (Hu et al., 2015). Pak choi can be harvested within 37 days, which was long enough to study the effects of DO on nitrogen transformations in aquaponic systems (Wongkiew et al., 2017b). (In the floating-raft aquaponic systems, rates of ammonia and nitrite oxidations reached their steady state within 7-10 days.) To study the effect of DO on nitrate accumulation, the author compared aquaponics without plants at low DO concentrations (3.0 ± 0.4 mg/L) with aquaponics without plants at high DO concentrations (6.9 ± 0.4 mg/L). No plants were used in this experiment to eliminate the interfering effects of nitrogen uptake by plants, and nitrate accumulation on nitrogen transformations.
- **Effect of DO on denitrification (using natural abundance $\delta^{15}N$).** Aquaponic systems without plants at high DO (DO: 6.91 ± 0.35 mg/L) and low DO (DO: 3.02 ± 0.35 mg/L) concentrations in fish tanks (inlet of biofilters) were run simultaneously and compared over 12 days. Since nitrate is the major reactant in denitrification process, water in the fish tanks was collected every three days for measurements of nitrate concentration and

$\delta^{15}N$ values of nitrate. In this experiment, plants were not grown in the aquaponic systems because nitrogen uptake by plants could affect nitrate and $\delta^{15}N$ values in recirculating water. The systems were operated at HLR of 1.5 m^3/m^2-day, and fish were fed at a constant feeding rate of 35 g/day. The recirculating waters of aquaponic systems were mixed thoroughly to obtain the same initial concentration of nitrate and $\delta^{15}N$ value of nitrate prior to the start of the plant-less experiment. Figure 3.3 shows a conceptual diagram of this experiment.

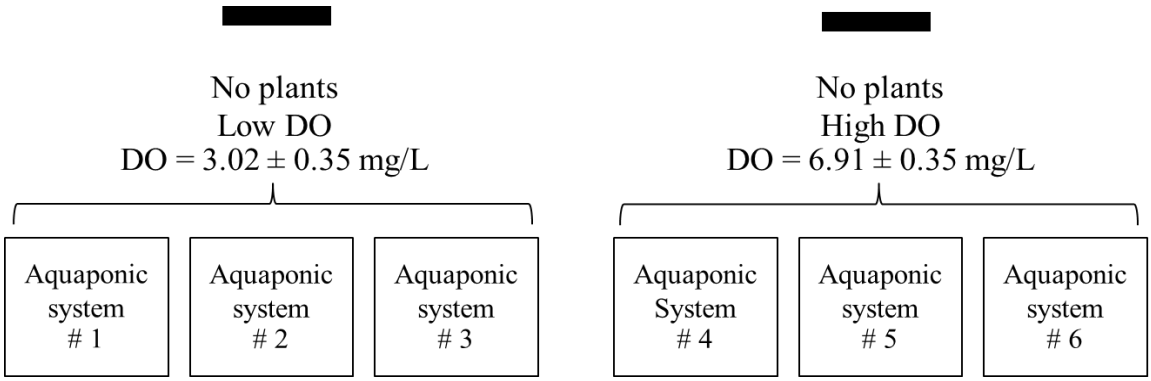

Figure 3.3. Experimental diagram for investigating nitrogen transformations at two DO levels (low DO vs. high DO) using natural abundance nitrogen isotopic compositions

- **Effect of HLR on nitrogen transformations:** Aquaponic systems were operated at varying HLRs (e.g., 0.10, 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 m^3/m^2-day) (Figure 3.2). Lettuce, pak choi, chive, and tomato-based aquaponic systems were selected to study the effect of HLRs on nitrogen transformations. Lettuce and pak choi were selected because they are widely consumed vegetables and grow well in the aquaponic systems with a harvesting time of about five weeks after transplanting to grow bed. Tomato is a fast-growing fruity plant that produces higher amount of roots and has higher root total surface area compared to pak choi and lettuce. Chive (slow-growing plant) is the species that produce a relatively small amount of root and root surface area among the four plant species. Since the author hypothesized that HLRs could affect nitrogen transformations differently in different plant-based aquaponic systems, evaluating the effect of HLR on nitrogen transformations in different plant-based aquaponic systems was necessary (Hu et

al., 2015). Thus, four plant species, namely lettuce, pak choi, tomato, and chive were selected to study the effects of HLR on nitrogen transformations.

- **Effect of pH on nitrogen transformations:** Lettuce- and chive-based aquaponic systems were used to study the effects of pH on nitrogen transformations. The author hypothesized that plants with different growth rates, affected by pH, could cause different nitrogen transformations in aquaponic systems (Hu et al., 2015; Zou et al., 2016b). Studies reported that pH affected a total root surface area of fast-growing vegetables (e.g., pak choi, lettuce, and tomato) and their yields in aquaponic systems (Zou et al., 2016b). However, due to a slow growth rate of chive, pH did not significantly affect the yield and the surface area of chive root as much as pak choi, lettuce, and tomato. Thus, both lettuce (fast-growing species) and chive (slow-growing species) were selected to grow in this experiment to elucidate the effects of pH on nitrogen transformations in two aquaponic systems that have different plant growth rates. In the chive-based experiment, TAN, nitrite and nitrate concentrations from the chive-based aquaponic systems operated at near neutral pH level (7.0 ± 0.2) were compared with those under two acidic pH levels (6.0 ± 0.2, 5.2 ± 0.2) for a month. For lettuce, nitrogen transformations in lettuce-based aquaponic systems at two different pH levels (6.1 ± 0.3 and 6.9 ± 0.3) were compared.

3.2.2. Examine the transformations of different forms of nitrogen in an aquaponic system under different conditions

Part 1: Nitrogen products distributions in aquaponic systems

All possible forms of nitrogen were monitored. Fish feed is the only nitrogen input in the aquaponic systems. There are many forms of nitrogen in the outputs, namely, fish muscle tissue, TAN, nitrite nitrogen, nitrate nitrogen, vegetable biomass, sediment accumulated in biofilters (fish feces and microbes), and nitrogen loss (N_2 and N_2O gas). Nitrogen product distributions in percentage relative to nitrogen in fish feed input (% nitrogen recovery) were calculated using nitrogen mass balance at the end of each experiment (see section 3.5 and Appendix B (Eqs. B1-B4)). All of the experiments in this part were run in triplicate.

- **Transformations of different forms of nitrogen in different plant-based aquaponic systems.** In this experiment, fish were fed with a constant feeding rate of 35 g/day. Aquaponic systems were operated at the condition that facilitates high nitrification efficiency (obtained from objective 1). The condition included DO concentration above 6.0 mg/L (aeration rate of 10 L/minute in fish tank), HLR of 1.5 m^3/m^2-day, and pH around neutral (pH ~6.7-7.2). Five types of aquaponics systems were operated, namely pak choi-, lettuce-, tomato- and chive-based aquaponic systems, and aquaponics with no plants. Figure 3.4 shows the experimental diagram of this experiment.

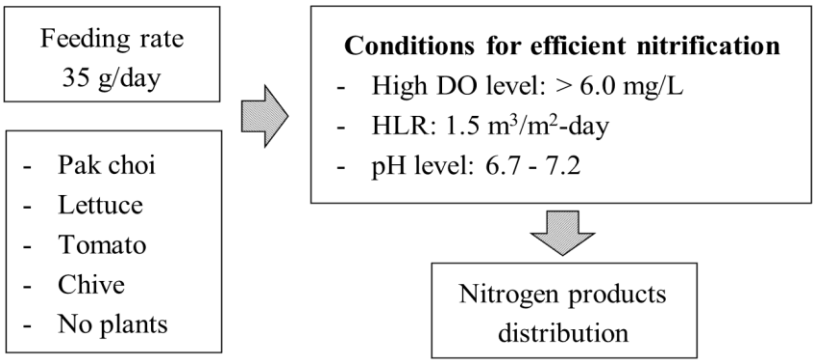

Figure 3.4. Experimental diagram for quantifying nitrogen products distribution in different plant-based aquaponic systems

- **Transformations of different forms of nitrogen at different feeding rates.** In this experiment, nitrogen inputs (feeding rates) were varied. Fish were fed with three constant feeding rates of 15, 35, and 50 g/day. Similar as the previous bullet, aquaponic systems were operated at the condition that facilitates high nitrification efficiency (DO > 6.0 mg/L, HLR of 1.5 m^3/m^2-day, pH around 6.7- 7.2). Lettuce was used in this study because it is a widely consumed vegetable and grows well in the aquaponic systems with a harvesting time of 32 days after transplanting to grow bed. Figure 3.5 shows the scope of this experiment.

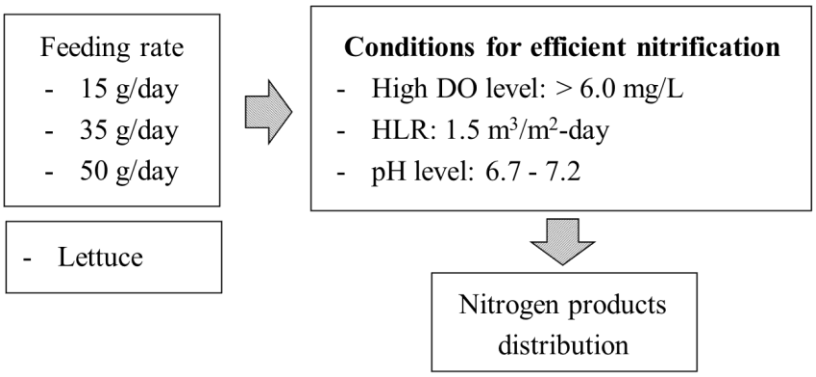

Figure 3.5. Experimental diagram for quantifying nitrogen products distribution at three different feeding rates

- **Transformations of different forms of nitrogen at different HLRs.** In this experiment, fish were fed with a constant feeding rate of 35 g/day. Aquaponic systems were operated at the DO concentration (DO > 6.0 mg/L), and pH levels around 6.7-7.2. Nitrogen product distributions from aquaponic systems operated at HLRs of 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 m^3/m^2-day were compared. Lettuce was used in this experiment due to the same reason as mentioned previously. Figure 3.6 shows the scope of this experiment.

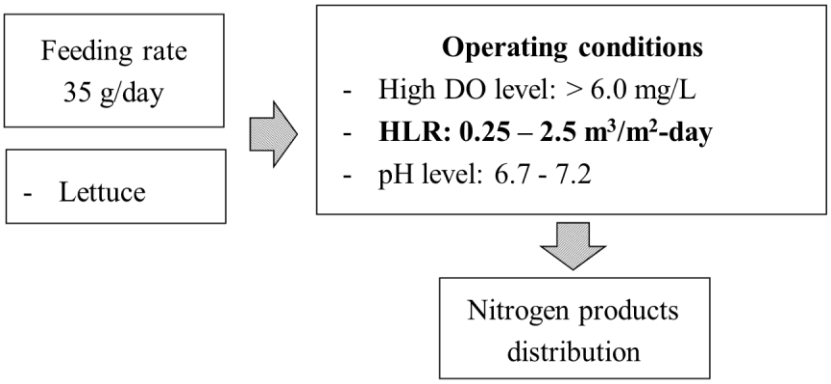

Figure 3.6. Experimental diagram for quantifying nitrogen products distribution at different hydraulic loading rates (feeding rate = 35 g/day)

An additional experiment was also designed to confirm the results that HLRs above the recommended levels (1.5 m^3/m^2-day, the results from the objective 1) would not make any difference on nitrogen products distribution at a higher feeding rate and

with other plant species. A constant feeding rate of 50 g/day and pak choi-based aquaponic systems were used in this experimental part. Aquaponic systems were operated at the DO concentration (DO > 6.0 mg/L) and pH levels around 6.7-7.2. Figure 3.7 shows the scope of this experiment.

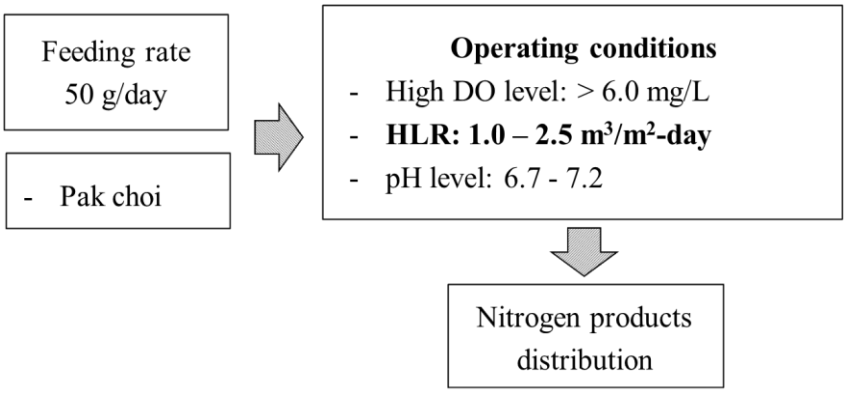

Figure 3.7. Experimental diagram for quantifying nitrogen products distribution at different hydraulic loading rate (feeding rate = 50 g/days)

- **Transformations of different forms of nitrogen at different DO levels.** In this experiment, aquaponic systems operated at two different DO levels in fish tanks (low DO = 3.97 mg/L and high DO = 7.44 mg/L) were compared. Fish were fed with a constant feeding rate of 35 g/day. Aquaponic systems were operated at the condition that facilitates high nitrification efficiency (HLR of 1.5 m^3/m^2-day, pH around 6.7- 7.2). Pak choi was used in this study because it is tolerable to low DO concentration (2-4 mg/L) and warm temperature (up to 30^oC) and was previously grown in aquaponic systems operated at low DO concentrations with no plant stress (Fang et al., 2017; Zou et al., 2016a). Figure 3.8 shows the scope of this experiment.

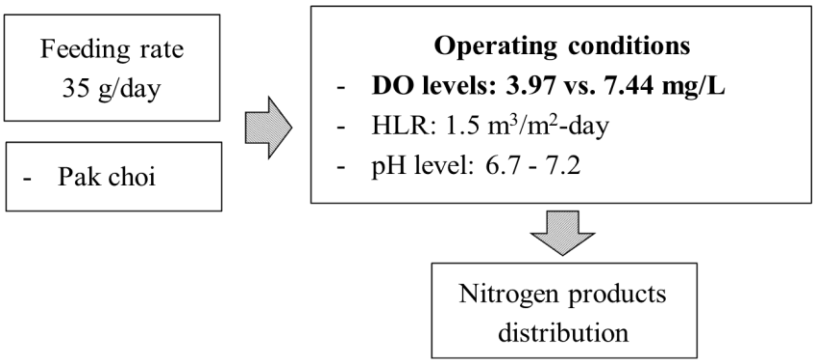

Figure 3.8. Experimental diagram for quantifying nitrogen products distribution at two DO levels (high DO vs. low DO)

Part 2: Nitrogen isotope studies in aquaponic systems

In this part, a fate of nitrogen in the floating-raft aquaponic systems was evaluated using natural abundance nitrogen isotope and labeling nitrogen isotope approaches. This part began with natural abundance nitrogen isotope (triplicate). Then the nitrogen transformations in aquaponics were studied in duplicate using enriched $K^{15}NO_3$ and $(^{15}NH_4)_2(SO_4)$. Combining the two nitrogen isotope approaches together, the fate of nitrogen was discussed.

- **Evaluation of natural abundance nitrogen isotopic composition ($\delta^{15}N$) values.** Baselines natural abundance nitrogen isotopic compositions in the aquaponic systems were evaluated by sampling and analyzing isotope values of all collectable input and outputs, namely fish feed, fish muscle tissues, fish feces, sediment, nitrate in recirculating water, organic nitrogen in roots, stems, leaves, and fruits, and nitrate (extracts) in roots, stems, leaves, and fruits. Section 3.3 describes a sampling method for the baseline natural abundance $\delta^{15}N$ values in the aquaponic systems. Section 3.4 describes isotope analytical methods.
- **Effects of plant species on nitrogen uptakes and denitrification (labeling isotope approach using an enrich $K^{15}NO_3$).** Two experimental runs were studied in this task (see Figures 3.9 and 3.10). Due to a limited number of the aquaponic systems, pak choi, lettuce, and control were studied in the first run. Tomato, chive, and control were studied in the second run. Aquaponic systems were run in duplicate. At the beginning of this experiment, $K^{15}NO_3$ was added to aquaponic systems. $^{15}NO_3$ was enriched in this

experiment because nitrate was expected as the main nitrogen source for nitrogen assimilation by plants and the major source of denitrification. The $\delta^{15}N$ value of nitrate in the water of aquaponic systems were enriched to around 1,000 ‰ (0.7299 at% ^{15}N) to overwhelm isotope effects during nitrogen transformations (see Tables B.1 and B.2 for how to calculate the dose of spiked $^{15}KNO_3$). Nitrate concentration was maintained at high concentrations to facilitate the plant growth. All aquaponic systems were operated at high DO concentrations for efficient nitrification (DO = 6.32 ± 0.24 mg/L in run no.1, DO = 6.44 ± 0.45 mg/L in run no. 2). The aquaponic systems in run no.1 were operated for 32 days. The aquaponic systems in run no.2 were operated for 70 days for chive-based aquaponic systems, and 90 days for tomato-based aquaponic systems. Water samples in the fish tanks were collected every 1-2 weeks for nitrate concentration, $\delta^{15}N$ values of nitrate, and TAN, nitrite, and COD concentrations. Whole plant tissues and nitrate extracts were collected every 1-2 weeks for nitrogen content and $\delta^{15}N$ values. Roots, root extracts, and sediment were collected at the end of each experiment for nitrogen content and $\delta^{15}N$ values. The systems were operated at HLR of 1.5 m^3/m^2-day, and fish were fed at a constant feeding rate of 35 g/day. Nitrogen isotopic mass balance was conducted at the end of each experiment. Figures 3.9 and 3.10 show conceptual diagrams of this experimental part.

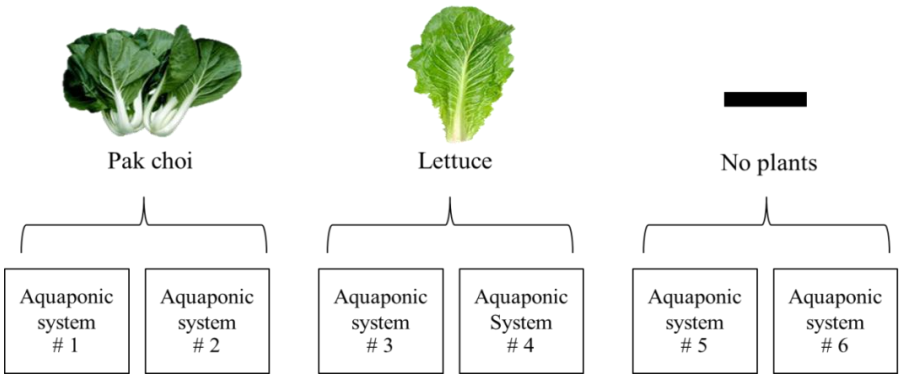

Figure 3.9. Experimental diagrams for investigating nitrogen transformations in different plant-based aquaponic systems (pak choi, lettuce, no plants) using enriched $K^{15}NO_3$

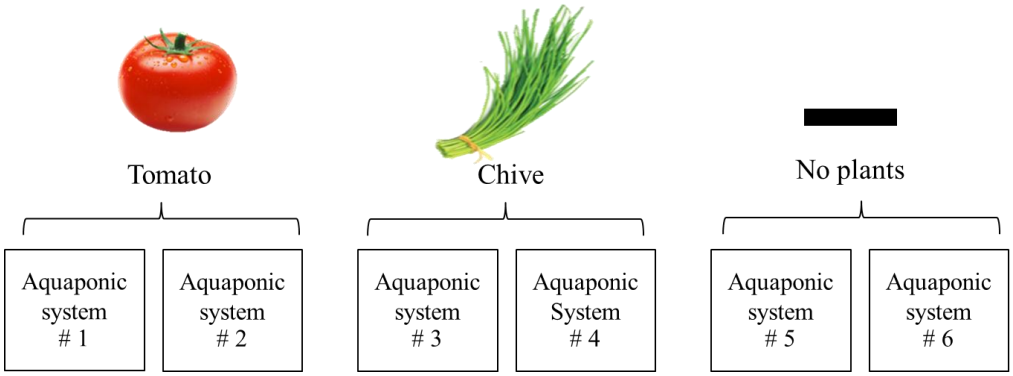

Figure 3.10. Experimental diagrams for investigating nitrogen transformations in different plant-based aquaponic systems (tomato, chive, no plants) using enriched $K^{15}NO_3$

- **Effects of DO on nitrification and denitrification (labeling isotope approach using an enrich $^{15}NH_4^+$).** Aquaponic systems without plants were operated in duplicate. At the beginning of this experiment, $(^{15}NH_4)_2(SO_4)$ was spiked to each aquaponic system. The $\delta^{15}N$ value of ammonium nitrogen in the water in aquaponic systems were hypothesized to increase above the baseline to around 2,000-3,000 ‰ (see Table B.1 for how to calculate the dose of spiked $(^{15}NH_4)_2SO_4$). There was no addition of $^{15}NO_3^-$ in this section. However, it was hypothesized that $^{15}NH_4^+$ could be oxidized to $^{15}NO_3^-$ rapidly. Initial nitrate concentrations and initial $\delta^{15}N$ values of nitrate in each aquaponic systems were maintained at low values (<6 mg N/L and ~16.4 ‰) to reduce an interference from the huge backgrounds of nitrate nitrogen and $\delta^{15}N$ values of nitrate. To evaluate the effect of DO on nitrogen transformations, aquaponics at high DO (DO: 6.85 ± 0.20 mg/L) and low DO (DO: 3.83 ± 0.47 mg/L) concentrations in the fish tank (inlet of biofilters) were run simultaneously with no plants and compared over 12 days. Since nitrate is the major reactant in denitrification process, water in the fish tanks was collected every two days for nitrate concentration, $\delta^{15}N$ values of nitrate, and TAN, nitrite, and COD concentrations. The systems were operated at HLR of 1.5 m^3/m^2-day, and fish were fed at a constant feeding rate of 35 g/day. Nitrogen isotopic mass balance (Eqs. B5 and B6) was conducted at the end of the experiment. Figure 3.11 shows a conceptual diagram of this experiment.

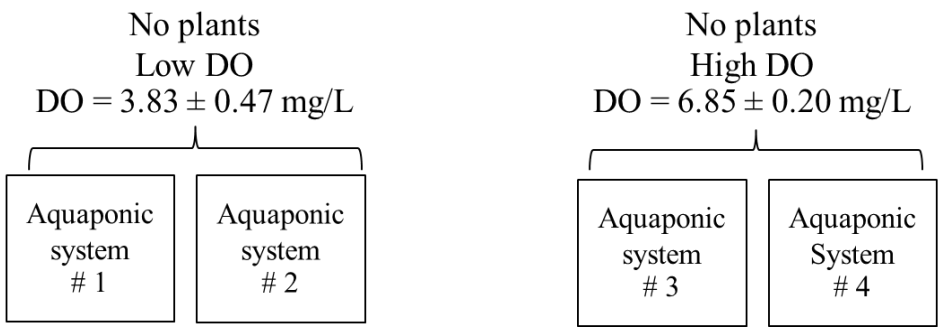

Figure 3.11. Experimental diagram for investigating nitrogen transformations at two DO levels (low DO vs. high DO) using labeling nitrogen isotope approach

3.2.3. Examine the ecology of functionally important living microbes and assess their contributions to nitrogen transformations in aquaponic systems

- **Microbial communities in different plant-based aquaponic systems.** Pak choi-, lettuce, tomato-, and chive-based aquaponic systems were operated at an operating condition for efficient nitrification with no accumulations of TAN and nitrite concentrations in the aquaponic systems. The operating condition was found from the results from objectives 1 and 2 (HLR of 1.5 m^3/m^2-day, DO >6.0 mg/L, and pH range of 6.7-7.2). Fish were fed with a constant feeding rate of 35 g/day once a day. At the end of each harvesting cycle of the four aquaponic systems, microbial samples (n = 3) were collected from the plant roots and the upflow biofilter (Figure 3.12), and stored at -80 ^oC for subsequent microbial community analysis. Samples from biofilter and plant roots were separately subjected to NGS and qPCR analyses (see sampling method in section 3.3 for details of sampling). In this part (objective 3), the term combined samples represent the samples from the upflow biofilter (see sampling method in section 3.3 for details of sampling).

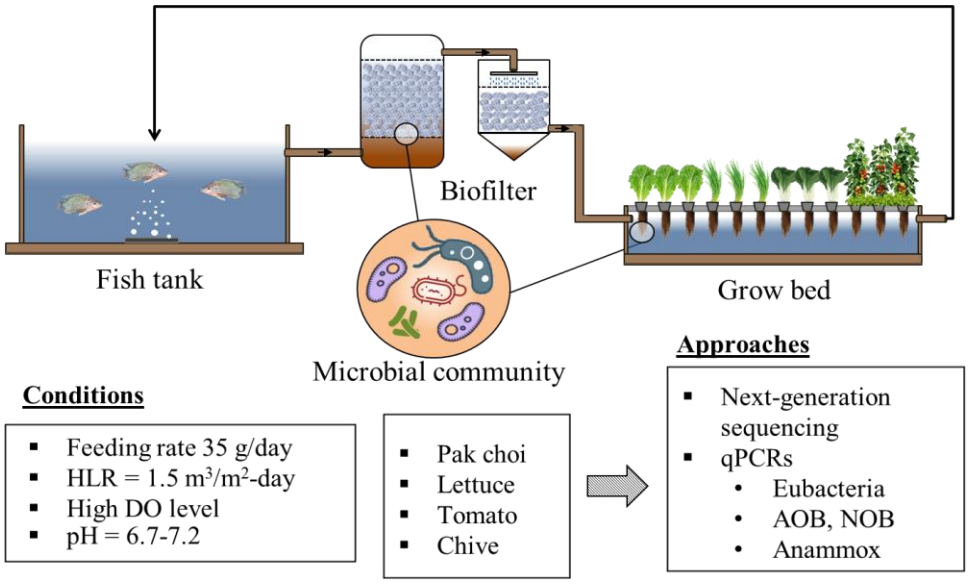

Figure 3.12. Experimental diagrams for examining microbial community in different plant-based aquaponic systems

- **Microbial communities at different pH levels.** Chive-based aquaponic systems operated in triplicate were used to study the effect of pH levels on microbial community. Three pH levels (5.2, 6.0, and 7.0 (near neutral)) were studied in this this experiment (Figure 3.13). The aquaponic systems were operated at an effective condition that promotes high nitrification efficiency. Operating parameters, feeding rate, and microbial sampling and preparation were the same as described previously in the microbial community in different plant-based aquaponic systems.

  DO concentrations in rhizosphere zone of grow beds were maintained at the same levels (DO = 5.8 ± 0.3 mg/L) at each pH condition to minimize the interference of DO level on microbial community, and plant and root growths (Chérif et al., 1997; Philippot et al., 2013; Vacheron et al., 2013). Previous studies reported that pH affected the growth of plant roots (Kang et al., 2011; Monshausen et al., 2007; Zu et al., 2014), and plant roots caused a depletion in DO concentrations around root zone via aerobic respiration of rhizobacteria (Hu et al., 2015; Philippot et al., 2013). As a result, different pH levels could indirectly contribute to a difference in DO concentrations around rhizosphere, especially in fast-growing plants. Different DO concentrations affected by different pH levels could bias the direct effect of pH on microbial communities in plant roots. Thus,

the use of fast-growing plants (e.g., lettuce, pak choi, and tomato) at two or more pH levels may lead to misinterpretation of microbial community shift whether such shift is caused by the pHs or the DOs. Thus, chive-based aquaponic systems were selected to evaluate the effects of pH on nitrogen transformations and microbial communities where growth of chive did not cause significant DO depletion in the grow bed thereby reducing the indirect effects of DO on microbial communities on roots (Park et al., 2017; Philippot et al., 2013; Vacheron et al., 2013). Samples from plant roots and biofilter under each plant species were separately subjected to NGS and qPCR analyses (Figure 3.13) (see sampling method in section 3.3).

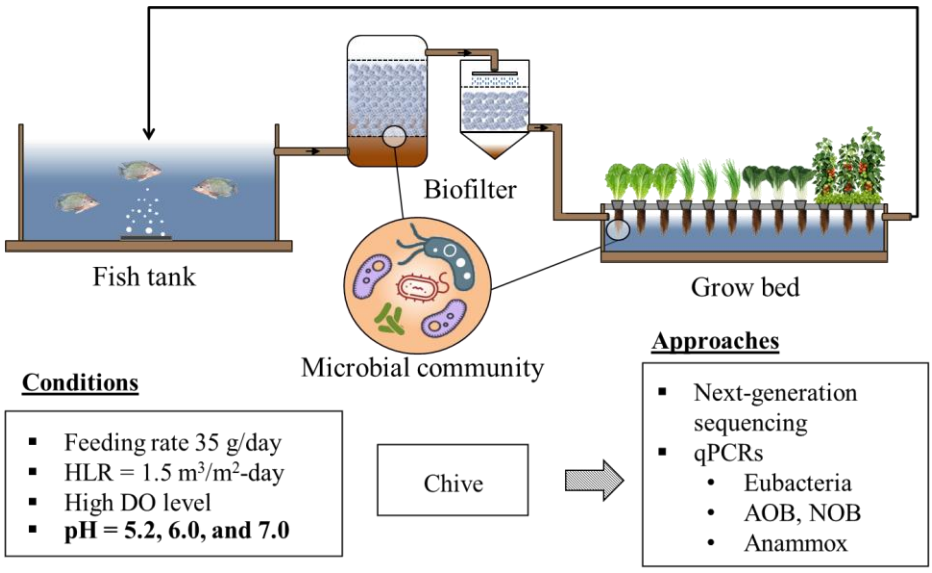

Figure 3.13. Experimental diagrams for examining the effect of pH on microbial community in chive-based aquaponic systems

3.2.4. Investigate the greenhouse gases emissions from an aquaponic system, with specific emphasis on nitrous oxide (N_2O) emission

In this objective 4, aquaponic systems were operated at an operating condition for efficient nitrification with no accumulations of TAN and nitrite concentrations in aquaponic systems. The operating condition was found from the results from objectives 1 and 2 (HLR of 1.5 m^3/m^2-day, DO >6.0 mg/L, and pH range of 6.7-7.2). Fish were fed with a constant feeding rate of 35 g/day, once a day. Nitrous oxide emissions in fish tanks (aerated surface) and in

biofilters (non-aerated surface) were measured separately (Figure 3.14). N_2O gas emissions were investigated at the end of each harvesting cycle (sampling size, n = 4). N_2O gas emission rate from an aquaponic system was calculated by summing N_2O gas emissions from a fish tank and biofilter (Figure 3.14).

In aerated surface, fish tanks were tightly covered and entirely sealed with a plastic sheet before gas sampling. The cover has one-way air entrance from air diffusers at the bottom of a fish tank and one-way air exit at the top of the fish tank. Gas samples were collected at a small hole located at the fish tank cover near the one-way gas outlet. Fish tanks were aerated with a constant flow rate of 10 L/minute. All fish tanks were covered for 30 minutes before gas sampling to ensure a constant N_2O concentration. Four gas samples of each fish tanks were analyzed for N_2O concentrations. An average value of the four samples was used as a representative of N_2O emission rate from a fish tank. N_2O emission rate from a fish tank (aerate surface) was calculated using Eq. 3.1 (Sun et al., 2013).

$$\text{Aerated surface:} \qquad E = Qc\rho \qquad (3.1)$$

Where E is N_2O emission rate (g/day), Q is air flow rate (m^3/day), c is the concentration of N_2O (10^{-6} m^3/m^3), and ρ is N_2O gas density at a temperature (g/m^3). The ideal gas law (pV = nRT) was used to determine ρ of N_2O at any temperature. The atmospheric pressure was assumed at 1 atm. Background N_2O concentration was subtracted from N_2O concentrations in gas samples before calculating the N_2O emission rate.

In non-aerated surface, the first biofilter (upflow biofilter) were tightly closed and sealed with a bucket lid O-ring. Gas was not allowed to exit directly from the first biofilter to the atmosphere (Figure 3.14). Gas exit of the first biofilter connected to the second biofilter (downflow with partial aeration, water drop system). Gas sampling was conducted at the headspace this downflow biofilter because the partial aeration allowed high turbulence between air-water interfaces that released N_2O gas from the water to the air (Figure 3.14). One-way air exit of the downflow biofilter was closed during the gas sampling to prevent the diffusion of N_2O from ambient air. N_2O emission rate from the downflow biofilter (non-aerate surface) was calculated using Eq. 3.2 (Sun et al., 2013).

Non-aerated surface: $E = (24x60)(dc/dt)\rho V$ (3.2)

Where E is N_2O emission rate (g/day), c is the concentration of N_2O (10^{-6} m^3/m^3), t is time (min), ρ = N_2O gas density at a temperature (g/m^3), V is volume of a chamber of biofilter (m^3), and dc/dt is a linear slope of N_2O concentrations increasing over time (minute) (at 0, 3, 6, and 9 minutes). The air in the second biofilter (downflow) was opened to ambient air at time 0 minute to reduce the N_2O accumulated in the biofilter. Then the second biofilter was immediately closed to allow the accumulation of N_2O gas in the biofilter, resulting in an increase in N_2O concentrations over time (dc/dt); at this moment, the 0 minute of gas sampling began. Figure 3.14 shows a diagram of gas sampling in an aquaponic system.

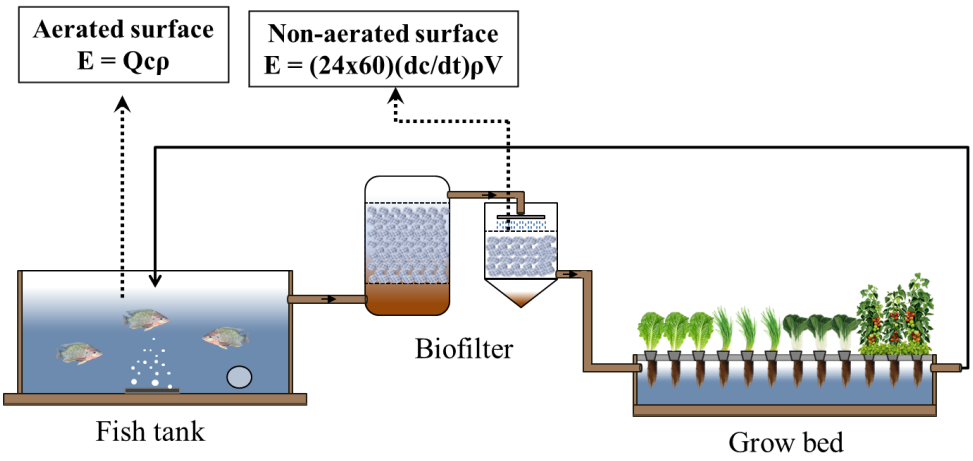

Figure 3.14. Schematic diagrams for sampling nitrous oxide gas from aquaponic systems

- **Nitrous oxide emissions from aquaponic systems.** Nitrous oxide emissions from pak choi-, lettuce-, tomato-, and chive-based aquaponics and aquaponics with no plants (control) were compared. Aquaponic systems were operated in duplicate. N_2O gas samplings were conducted at the end of each harvesting cycle (n = 4). There were two experimental runs in this part. At run no. 1, pak choi- and lettuce-based aquaponic systems and aquaponics with no plants were run simultaneously in parallel for 35 days. At run no.2 tomato- and chive-based aquaponic systems and aquaponics with no plants were run simultaneously in parallel for 90 days. Figure 3.15 shows a conceptual design of this experiment.

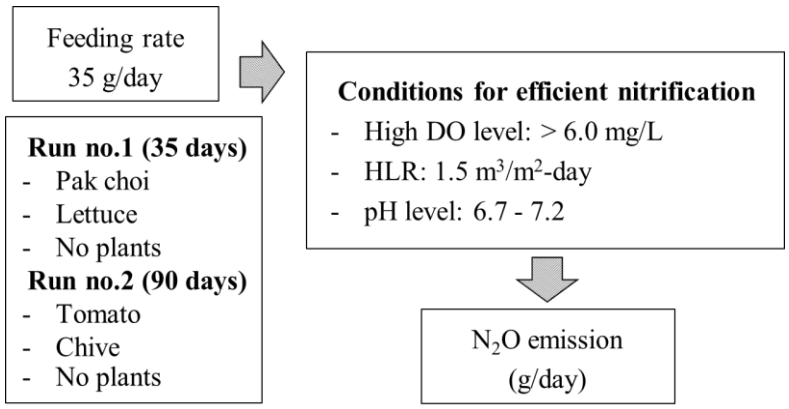

Figure 3.15. Schematic diagrams for investigating nitrous oxide emissions from different plant-based aquaponic systems

- **Development of strategies to reduce nitrous oxide emissions.** Two strategies were selected to reduce N_2O emission from the aquaponic systems. The strategy one was to aerate the first biofilter in which high rates of denitrification and N_2O emission were expected to occur. This strategy could increase the DO levels in the biofilter and reduce N_2O emission (Hu et al., 2012; Lu et al., 2014b). Aeration rate of 1 L/minute was supplied at the bottom part of the first biofilter over an operating time of an aquaponic system. Addition of N_2O emission rate from this aerated biofilter (the upflow biofilter) was included when calculating N_2O emission from aquaponic systems (aerated surface, Eq. 3.1). The strategy two was adding effective microorganism (EM), a commercially available organic soil-microbial inoculant, to aquaponic systems. It was hypothesized that EM addition could improve water quality, which could lead to a lower N_2O emission rate (Majumdar, 2003; B. Wang et al., 2016). One ounce of EM (EM•1 Microbial Inoculant™, OMRI, OR) per gallon of water was added to an aquaponic system weekly. The two mitigation strategies and one control were run in parallel in duplicate, and N_2O emissions from two strategies were compared with N_2O emissions from the control at the end of each harvesting cycle. N_2O gas samplings were conducted at the end of each harvesting cycle (n = 4). Figure 3.16 shows a conceptual design of this experiment.

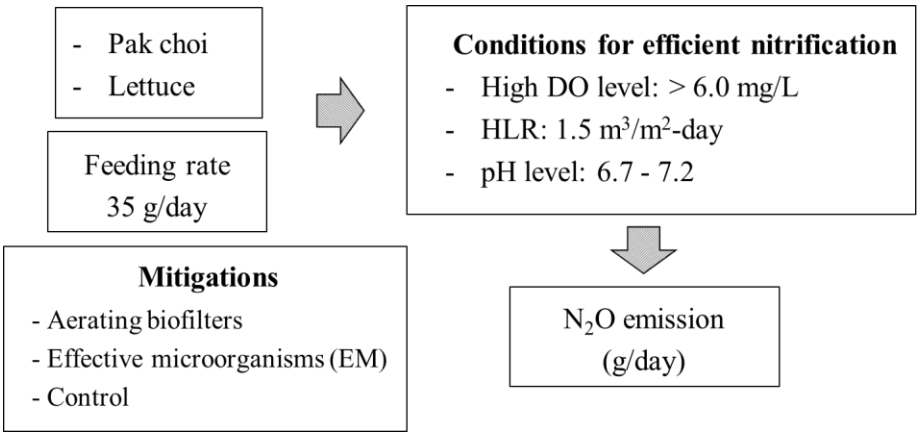

Figure 3.16. Experimental diagram and mitigation strategies to reduce nitrous oxide emissions from aquaponic systems

- **Effect of feeding rates on nitrous oxide emissions.** Lettuce-based aquaponic systems at two feeding rates of 15 and 35 g/day were run in parallel for 32 days. N_2O emissions from two feeding conditions were compared. Aquaponic systems were operated in triplicate. N_2O gas samplings were conducted at the end of each harvesting cycle (n = 6). Figure 3.17 shows a conceptual design of this experiment.

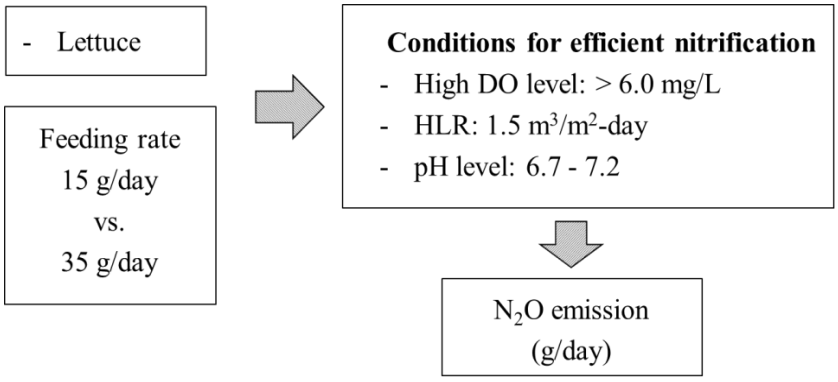

Figure 3.17. Experimental diagram comparing the effects feeding rates (15 g/day vs. 35 g/day) on nitrous oxide emissions from aquaponic systems

3.3. Sampling methods

DO, pH and temperature were monitored daily in fish tanks and grow beds. Water samples were collected weekly from fish tanks representing the whole recirculating water in the aquaponic systems. The water samples were analyzed for total Kjeldahl nitrogen (TKN), TAN, nitrite, nitrate, and COD (for chemical analyses, see section 3.4.1 and Table A.1). At the end of each experiment, samples (n = 3) of the stems, roots, leaves, fruits (tomato), whole plants (stems and leafs), settled sediment in the biofilters, and feed were dried at 60 ^{o}C, and then analyzed for TKN. TAN, nitrite and nitrate contents in the feed, and fish muscle tissue were negligible due to their low nitrogen contents compared to organic nitrogen (Hu et al., 2015). At the end of all experiments, samples of fish muscle (n=3) tissue were collected, dried at about 60 ^{o}C and analyzed for organic nitrogen content. Thus, TKN concentrations in fish feed, fish muscle tissue, and plants were assumed to be equal to organic nitrogen content.

For isotope analyses (objective 2), the solid samples of fish feed, fish muscle tissue, fish feces, sediment from the biofilters, plant roots, stems, leaves, whole plants, and fruits (tomato) were collected at the end of experiments (n=3) (section 3.2.2 part 2), and were dried at 60 ^{o}C, ground and homogenized using a mortar and pestle. Stems, leaves, roots, whole plants, and fruits were also extracted for nitrate using a mortar and pestle, and stored at -80 ^{o}C for subsequent analysis.

For microbial analyses (objective 3, section 4.3), at the end of each experiment, microbial samples (n = 3) were collected from biofilm of biofilters, roots, fish tank effluent, and settled sediment in the upflow biofilters, and stored at -80 ^{o}C for subsequent microbial community analysis. Because the aquaponic upflow biofilter consists of three subcomponents (biofilm, fish tank effluent, and settled sediment), the microbial community representing a biofilter was determined by combining raw samples from the biofilm, the fish tank effluent, and the settled sediment. Therefore, "combined sample" in this study represented "mixed sample from upflow biofilter", which were the combination of the biofilm, fish tank effluent, and settled sediment. In the aquaponic systems (Figure 3.1), downflow biofilter was not designed to function as biofilter. The downflow biofilter was designed for partial aeration (see section 3.1). Thus, combined samples represent only upflow biofilter in this study. The combined and root samples were

subjected to NGS analyses. For further insights on specific microbial community, the samples from roots and each subcomponent of biofilters (biofilm, fish tank effluent, and settled sediment) under each operating condition were separately subjected to qPCR analysis. Analytical details for microbial analyses can be found in sections 3.4.3 and 3.4.4.

3.4. Analytical methods

3.4.1. Chemical and physical analyses

DO, pH, and temperature in the fish tanks and grow beds were monitored using the HQ40d Portable Water Quality Lab Package (HACH, Loveland, CO, USA). TKN, TAN, nitrite, nitrate, and COD were analyzed weekly using a HACH Digesdahl digestion method (HACH 8075, Loveland, CO, USA), Nessler method (HACH 8038), NitriVer® 3 diazotization method (HACH 8507), Dimethylphenol method (HACH 10206), and Reactor Digestion method (HACH 8000), respectively. The total solids (TS) concentration of the accumulated sediment in biofilters was determined according to Standard Methods (Rice et al., 2012). Moisture contents were determined using a moisture analyzer (MOC-120H, Shimadzu, Japan). The dry weight of vegetable and fish biomass yields were calculated based on moisture content. The total nitrogen yield of the vegetables and fish (represented by fish muscle tissue) were calculated based on nitrogen content (mg N/kg biomass dry weight) in each dry sample. Plant root surface areas were determined using a root scanner integrated with WinRHIZO software (WinRhizo Pro v.2005b, Régent Instruments, Québec, Canada). Gas samples were collected from fish tank and biofilter for N_2O analysis using Gas Chromatography-Electron Capture Detector (GC-ECD) (Shimadzu GC-2014, Shimadzu, Japan). Oxygen uptake rate (OUR) of the sediment in biofilters was determined using an assembly consisting of a YSI 5100 DO meter and a self-stirring DO probe (YSI 5100 Dissolved Oxygen Instrument, OH, USA). The specific oxygen uptake rate (SOUR) of the sediment was then calculated using the OUR divided by volatile suspended solid (VSS) concentration, which was determined according to Standard Method (Rice et al., 2012).

3.4.2. Nitrogen isotopic composition ($\delta^{15}N$)

The nitrogen contents and $\delta^{15}N$ values of the samples were determined using an isotope ratio mass spectrometer (IRMS; Delta ^{Plus}XP, Bremen, Germany) coupled to an elemental

analyzer (Conflo IV/Costech ECS 4010) (Wongkiew et al., 2017b). Isotope values are reported in conventional *δ*-notation relative to the international standards atmospheric N_2 for N. The $\delta^{15}N$ value of nitrite and nitrate in the recirculating water, water extracted from stems, leaves, whole plants, and fruits were also analyzed; however, nitrate was the major form of dissolved inorganic nitrogen (DIN) in the aqueous phase. The $\delta^{15}N$ value of N_2O produced from nitrite + nitrate using the 'denitrifier method' (Sigman et al., 2001) with *Pseudomonas aureofaciens* cultures, was measured using automated methods described in Casciotti et al. (2002) followed by quantification of the masses of N_2O using an isotope ratio mass spectrophotometer (Finnigan MAT 252, Bremen, Germany).

3.4.3. Quantitative polymerase chain reaction (qPCR)

DNA from plant roots and other aquaponic samples were extracted using DNeasy® plant mini kit (Qiagen, Valencia, CA, USA) and DNeasy Blood & Tissue kit (Qiagen, Inc., Germantown, MD, USA), respectively. NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the extracted DNA concentrations and quality. The abundances of AOB, *Nitrospira* spp., *Nitrobacter* spp., and anammox bacteria (AMX) were quantified via SYBR® Green chemistry qPCR using specific primers (Table A.2), targeting *amoA* (Park et al., 2010; Rotthauwe et al., 1997), *Nitrospira* 16S rRNA (Graham et al., 2007; Park et al., 2010), *Nitrobacter* 16S rRNA (Graham et al., 2007; Park et al., 2010), and AMX 16S rRNA (Park et al., 2010; van der Star et al., 2007). Total bacterial abundance was quantified using eubacterial 16S rRNA targeted primers (Ferris et al., 1996; Park et al., 2010) (Table A.2). The qPCRs were performed in duplicate on iQ5 real-time PCR thermal cycler and analyzed with iCycler iQTM software (Bio-Rad Laboratories, Hercules, CA, USA). Standard curves for qPCR were generated via serial decimal dilutions of plasmid DNA and primer specificity.

3.4.4. Next-generation sequencing and sequence analysis

The extracted DNA (as discussed in section 3.4.3) was purified using QIAquick DNA Cleanup kit with QIAcube (Qiagen, CA). Before sequencing, bacterial 16S rRNA gene in each sample was amplified using the same universal primers as used in qPCR (1055F/1329R) and

barcoded fusion primers with sequencing adaptors. NGS analysis was performed using Ion Torrent 318v2 Ion Chip and run on an Ion Torrent PGM (Thermo Fisher, MA) according to manufacturer's instructions (Ion PGM Hi-Q Sequencing Kit, Product No. MAN0009816). For bioinformatics analyses using Mothur ver. 1.36.1 (Schloss et al., 2009), reads were filtered with Phred score of 20, minimum sequence length of 300 bp, and maximum homopolymeric region length of 8 bp to ensure high quality for downstream data analysis. The filtered reads were aligned against the latest SILVA ribosomal database nr ver. 128 and taxonomically classified after removal of chimeric alignments. Sequence reads representing chloroplast DNA (class, order, and genus of chloroplast) from plant roots (Schmautz et al., 2017) were removed after taxonomy classification of operational taxonomic units (OTUs) before calculating final bacterial relative abundance. R software ver. 3.4.0 was used to generate heatmaps ((packages: vegan ver. 2.4-3) (Oksanen et al., 2017), gplots (Warnes et al., 2016), Heatplus (Ploner, 2015), and RColorBrewer (Neuwirth, 2014)), canonical correspondence analysis (CCA) plots (package: vegan ver. 2.4-3), coverage (package: entropart (Marcon and Herault, 2015)), and microbial diversity indices (package: vegan ver. 2.4-3).

3.5. Nitrogen isotope mass balance calculation

At the end of each harvesting cycle for all four plant species and no plants, nitrogen mass balances in aquaponic systems were conducted based on the rates of nitrogen input (N in fish feed) and nitrogen in the products (N in fish, plants, sediment, nitrogen loss, and N accumulation in water) (see Eq. 3.3, see Eqs. B1 to B4 for more details).

The nitrogen budget can be expressed as the rate of nitrogen changed during the period when the fish was fed (nitrogen input) until the products were harvested or generated (nitrogen outputs) as:

$$f_N.M_f = \frac{d}{dt}(C_{TAN} + C_{NO2\text{-}N} + C_{NO3\text{-}N} + C_{org\text{-}N})V + N_{veg}/T + N_{fish}/T + N_{sed}/T + N_{loss}/T \quad (3.3)$$

Where, f_N is the fraction of nitrogen in fish feed (gN/g); M_f is the feeding rate (g/day); C_{TAN}, $C_{NO2\text{-}N}$, $C_{NO3\text{-}N}$, and $C_{org\text{-}N}$ are the TAN, nitrite, nitrate, and organic nitrogen concentrations in recirculating water (gN/L), respectively; V is the volume of recirculating water (L); N_{veg} is the

average nitrogen gained in vegetables at the end of each experiment (harvesting cycle) (gN); N_{fish} is the average nitrogen in fish muscle tissue (gN); T is time (days) for each harvest; N_{sed} is the nitrogen in sediment accumulated in biofilters at the end of each batch (gN); and N_{loss}/T is the rate of gaseous nitrogen loss (gN/day). In this study, N_{loss}/T was unknown and was calculated by subtracting the nitrogen in the fish feed (left side of the Eq. 3.3) from the rest of known nitrogen products.

Nitrogen use efficiency (NUE, %) of vegetable and fish were calculated using Eq. 3.4:

$$NUE_{total} = NUE_{veg} + NUE_{fish} = (N_{veg}/(f_N.M_f) + N_{fish}/(f_N.M_f)) \text{ x}100 \qquad (3.4)$$

Where, NUE_{veg} and NUE_{fish} are the nitrogen use efficiency of vegetables and fish, respectively; NUE_{total} is the total nitrogen use efficiency of vegetables and fish. In this study, NUE_{veg} is an important parameter used to assess the efficiency of the aquaponic system in converting nitrogen in aquaculture effluent into outputs (vegetables). Therefore, overall NUE is represented as NUE_{veg} in this study if NUE_{fish} is not mentioned.

Isotope mass balance was calculated using Eq. 3.5 (Hayes, 2004) (see Eqs. B5 to B6 for more details). Since the total isotopic abundance is conserved in one system (Eq. 3.5), the author hypothesized that the Σ (N mass x $\delta^{15}N$) of precursors in our aquaponic systems equals the Σ (N mass x $\delta^{15}N$) of products. Isotopic fractionation value (ε) (Eq. 3.6), a change in $\delta^{15}N$ between two phases, was used to solve with Eq. 3.5 when two unknown need to be solved (Hayes, 2004).

$$\Sigma (N \text{ x } \delta^{15}N)_{before} = \Sigma (N \text{ x } \delta^{15}N)_{after} \qquad (3.5)$$

$$\varepsilon_{b\text{-}a} = [\{(\delta^{15}N_b+1000)/(\delta^{15}N_a+1000)\}\text{-}1] \text{ x } 1000 \qquad (3.6)$$

3.6. Statistical analysis

Statistical analyses were carried out using a 2-sample Student's *t* test (between two groups, $\alpha = 0.05$) or an analysis of variance (one-way ANOVA) (between multiple groups, $\alpha = 0.05$) followed by Tukey-Kramer post-test (Tukey, 1949) for identifying significant difference ($p < 0.05$) in Minitab 16 Statistical Software (Minitab 16.1.1, Minitab Inc.). Slope regression analysis statistical tests ($\alpha = 0.05$) were used to evaluate the trend of nitrogen concentrations (e.g., accumulation or depletion of nitrogen species) ($p < 0.05$).

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Quantify the impact of physical and chemical variables that regulate nitrogen transformations in aquaponic systems

4.1.1. Effect of DO on nitrogen transformations

To investigate the effects of DO on nitrogen transformations, TKN, TAN, nitrite, nitrate, and COD from pak choi-based aquaponics at both low DO and high DO conditions were compared (Table 4.1). DO concentration was found to be a significant factor affecting nitrification and denitrification. DO concentrations in fish tank did not significantly affect TKN, TAN, nitrite, nitrate, and COD concentrations at HLR of 1.5 m^3/m^2-day. Nitrite oxidation, however, was affected by low DO condition in fish tank at HLR of 1.0 m^3/m^2-day, as evident from an increase in nitrite concentration (Table 4.1, Run no.2). This could be due to low DO level associated with low HLR (1.0 m^3/m^2-day). DO concentration decreased in the upflow biofilter due to a high specific oxygen uptake rate (SOUR) of the sediment of $5.8 \pm 0.4 \times 10^{-4}$ mg DO/mg VSS-hour (n = 6). Oxygen transfer rate in the biofilter at HLR of 1.5 m^3/m^2-day was high enough to maintain sufficient DO concentration for nitrite oxidation despite the low DO concentration (3.97 ± 0.94 mg/L) in the fish tank (input of biofilter). In this experiment DO concentrations in fish tanks could not be maintained below 3.0 mg/L because this threshold level stresses fish metabolism and reduces their feed consumption rate at 35 g/day (T. Popma and Masser, 1999). To further support that HLR affected nitrite via the association of DO, the aquaponic systems were also operated at HLR ranging from 0.10 to 2.5 m^3/m^2-day (see section 4.1.3).

Nitrate accumulation occurred in the aquaponic systems with plants (Table 4.1) and without plants (Figure 4.1). The nitrate accumulation rates in aquaponics with no plants can be described well assuming zero-order kinetics (Figure 4.1). Nitrate accumulation rate was significantly affected by DO level. Nitrate accumulation rate at high DO (6.9 ± 0.4 mg/L) level was also higher than that at low DO (3.0 ± 0.4 mg/L) level by 30.3% (Figure 4.1) at a constant feeding rate. The lower nitrate accumulation at low DO level in aquaponics with no plants can be

explained by an increase in rate of nitrogen loss via denitrification. Therefore, nitrate accumulation was important to account for nitrogen products distributions and percent nitrogen recovery. TAN, nitrite and organic nitrogen in the aquaponic systems were negligible in the nitrogen products distribution due to their low and stable concentrations.

Table 4.1. Summary of nitrogen and COD concentrations in pak choi-based aquaponic systems operating at different DO levels.

Run no.	HLR (m^3/m^2-day)	DO in fish tanks (mg/L)	TKN (mg N/L)	TAN (mg N/L)	NO_2^- (mg N/L)	NO_3^- accumulation rate (mg N/L-day)	COD (mg/L)
1	1.5	3.97 (0.94)	3.9 (1.2)	1.07 (0.16)	0.24 (0.10)	0.69 (0.21)	77.3 (4.4)
		7.44 (0.29)	4.2 (1.4)	1.09 (0.15)	0.25 (0.07)	0.82 (0.23)	76.3 (5.5)
2	1.0	3.45 (0.74)	7.3 (2.3)	1.16 (0.35)	0.79 (0.27)[a]	0.39 (0.16)	95.2 (11.7)
		7.05 (0.37)	7.0 (1.7)	0.82 (0.33)	0.28 (0.18)[b]	0.48 (0.22)	93.2 (11.6)

(Note: Values are the mean of multiple data (n = 15) for TKN, TAN, NO_2^- and COD concentrations, n = 3 for NO_3^- accumulation rate, (n = 37) for DO concentrations, and values in parenthesis represent standard deviation. The superscripts a and b represent statistically different ($p < 0.05$) between two DO levels.)

To support the effect of DO on nitrogen loss via denitrification, the author compared the actual nitrate accumulation with a hypothetical nitrate accumulation in the absence of denitrification (modeled by Eq. B8 (Appendix B)), shown in Figure 4.2a (low DO level) and Figure 4.2b (high DO level). The results suggest that nitrification could occur simultaneously with denitrification at micro-environment (anoxic) in the biofilm (Yin et al., 2015) and the sediment in biofilters, but nitrification rate was higher than denitrification rate (Figures 4.2a and 4.2b). To support the coexistence of nitrate accumulation and denitrification, nitrate concentrations at both low and high DO conditions in the absence of feed using Eq. B10 (Appendix B) were modeled (Figures. 4.2a and 4.2b). The decreases in nitrate concentration over time supported the nitrogen loss via denitrification in aquaponic systems. In conclusion,

nitrification rate following ammonification by fish with simultaneous nitrification was higher than denitrification rate in the aquaponic biofilters, leading to nitrate accumulation as the main source of nitrogen (Feng et al., 2012; Lin et al., 2015; Semerci and Hasilci, 2016). To confirm and elucidate the denitrification process in aquaponic systems, changes in the $\delta^{15}N$ values of nitrate were studied, and results shown in next section (4.1.2). Microbial community profiles, enriched ^{15}N study, and N_2O emissions also support the occurrence of denitrification (see sections 4.1.2, 4.2.9, 4.2.11, 4.3, and 4.4 for results supporting the occurrence of denitrification).

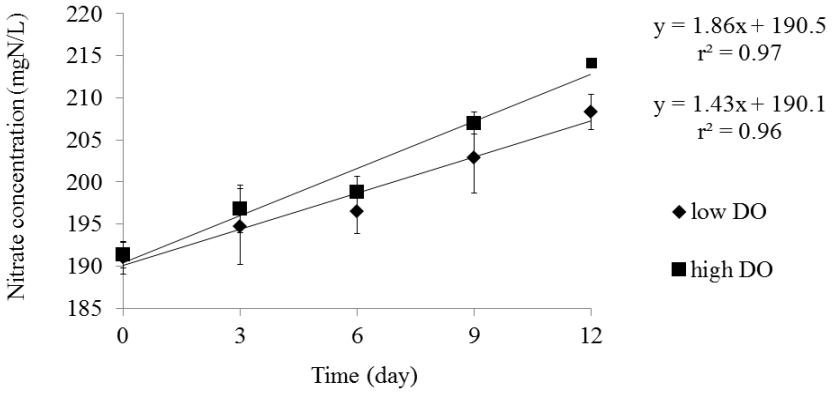

Figure 4.1. Nitrate concentrations in aquaponic systems with no plants at low DO (3.0 mg/L) and high DO (6.9 mg/L) conditions

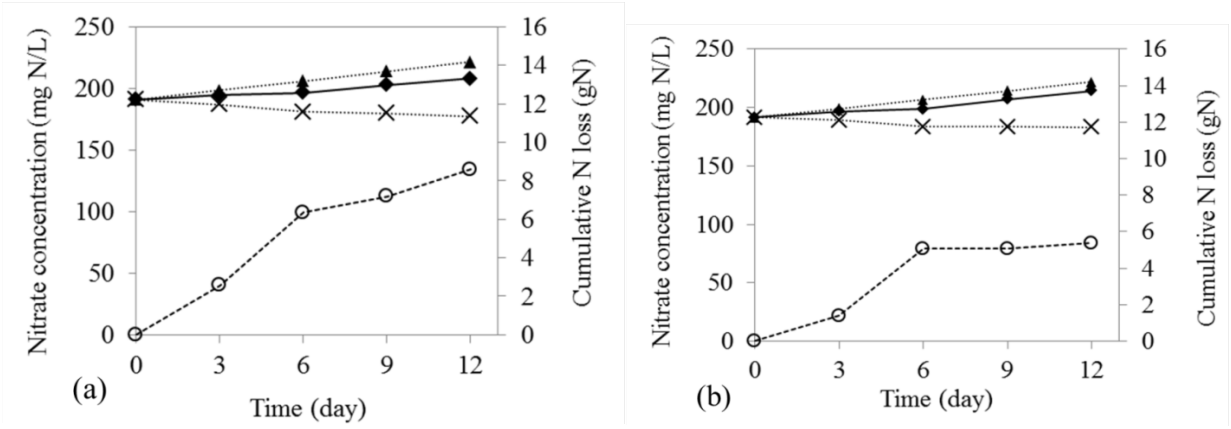

Figure 4.2. Modeled nitrate concentrations at low DO (3.0 mg/L) (a) and high DO (6.9 mg/L) conditions (b). Measured values (diamond with solid line) were compared with the modeled values in the absence of denitrification (triangle with dot line) and modeled values in the absence of daily feed (cross with dot line). Trends of cumulative N loss (circle with dash line)

4.1.2. Effects of DO on denitrification (using natural abundance $\delta^{15}N$)

Effect of DO on denitrification was investigated by nitrogen isotope mass balance. As hypothesized according to kinetic isotope effect (KIE), the $\delta^{15}N$ of nitrate will not be identical between denitrified water and non-denitrified water. Therefore, $\delta^{15}N$ values of nitrate in the aquaponic systems at low and high DO conditions would not be identical if the two denitrification rates were different. To investigate the effect of DO on denitrification rate, the author compared the $\delta^{15}N$ values of nitrate over 12 days between aquaponic systems under low DO (3.0 ± 0.4 mg/L) and high DO (6.9 ± 0.4 mg/L) levels with no plant growth since the efflux of nitrate from the plant roots could increase the $\delta^{15}N$ value of recirculating water (Evans, 2001). It was hypothesized that low DO promotes denitrification in the biofilter, and the $\delta^{15}N$ values of nitrate at the low DO level will be higher than the values at high DO due to greater loss of ^{15}N-depleted N_2. Therefore, denitrification under low DO level in the aquaponic system will cause higher $\delta^{15}N$ values of nitrate compared to $\delta^{15}N$ values of nitrate under high DO level.

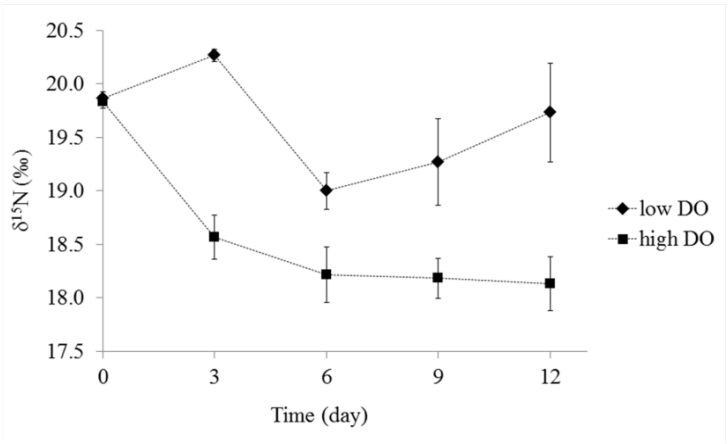

Figure 4.3. $\delta^{15}N$ values of nitrate in aquaponic systems with no plants at low DO (3.0 mg/L) and high DO (6.9 mg/L) conditions

Over 12 days, the aquaponic systems under low DO level generated higher nitrogen loss (29.6% of nitrogen input) than the aquaponic systems under high DO (18.5% of nitrogen input) (circles in Figures. 4.2a and 4.2b; cumulative N loss was calculated by Eq. B9). Denitrification resulted in the enrichment of ^{15}N in residual nitrate in the aquaponic systems and caused the increase in $\delta^{15}N$ values of nitrate due to the loss of ^{15}N-depleted N_2. According to the hypothesis, higher $\delta^{15}N$ values of nitrate indicated higher denitrification rate at low DO levels (Figure 4.3). Thus, $\delta^{15}N$ values of nitrate at high DO level suggest much lower rates of denitrification (Figure 4.3), which cause higher rate of nitrate accumulation (Figure 4.2). The author also modeled two extreme conditions using nitrogen isotope mass balance to support that the increases in $\delta^{15}N$ values of nitrate were due to denitrification (Figure 4.4).

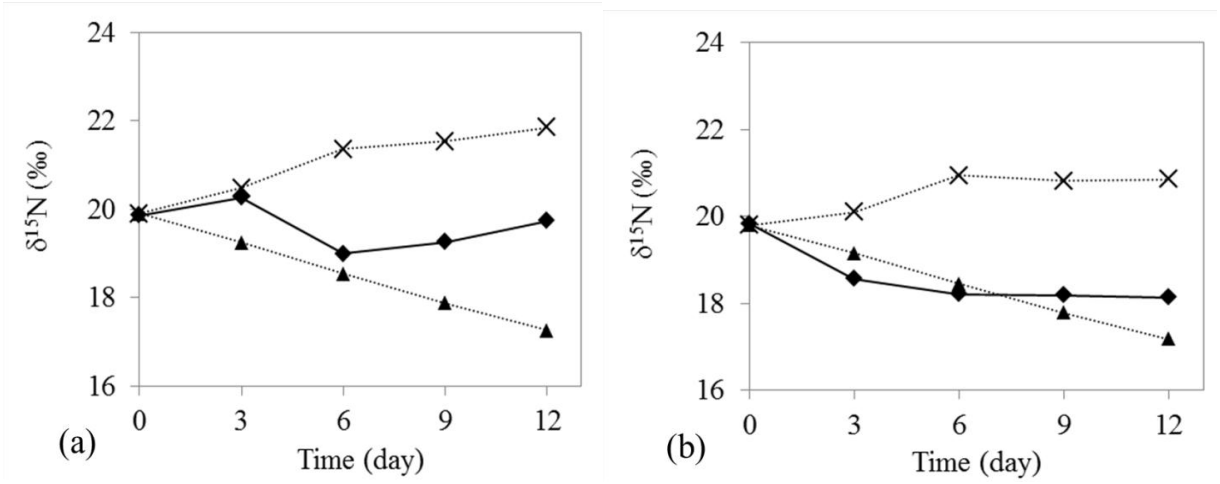

Figure 4.4. Modeled $\delta^{15}N$ values of nitrate at low DO (3.0 mg/L) (a) and high DO (6.9 mg/L) (b) conditions. Measured values (diamond with solid line) were compared with the modeled values in the absence of denitrification (triangle with dot line) and modeled values in the absence of daily feed (cross with dot line). Trends of cumulative N loss (circle with dash line)

In model 1, the author assumed daily input of fish food with a $\delta^{15}N$ value of 5.2 ± 0.5‰ and no denitrification (triangles in Figure 4.4, modeled by Eq. B12 (Appendix B)). If no denitrification was present, $\delta^{15}N$ values of nitrate in recirculating water would have been decreasing over the time and eventually approached the $\delta^{15}N$ value of the input (5.2 ± 0.5‰). Daily feeding decreased the measured $\delta^{15}N$ values of nitrate in the aquaponic systems because the $\delta^{15}N$ value in the fish feed ($\delta^{15}N$ = 5.2 ± 0.5‰) was lower than the $\delta^{15}N$ of nitrate in recirculating water ($\delta^{15}N_{feed} < \delta^{15}N_{water}$, see Figures 4.4a and 4.4b). In model 2, the author assumed the absence of daily fish feed with the same denitrification as occurred. A model assuming no input of feed during the experiment predicts a decrease in nitrate concentration and an increase in the $\delta^{15}N$ values of nitrate over time due to denitrification (crosses in Figures 4.4a and 4.4b, modeled by Eq. B13). Measured $\delta^{15}N$ values of nitrate over time fall between these two extremes (models 1 and 2), suggesting at least some denitrification occurred in the aquaponics operating at DO concentrations of 3.0 to 6.9 mg/L.

In conclusion, the isotope study showed that higher nitrogen loss in aquaponic systems was due to the denitrification that was enhanced under low DO concentration. In other

recirculating aquaculture wastewater systems, the nitrification and partial denitrification were reported to predominantly occur in biofilters, and DO was an important factor for nitrification and denitrification (Ge et al., 2015; Malone and Pfeiffer, 2006; von Ahnen et al., 2015). This suggests that nitrogen loss via denitrification could be reduced by maintaining aerobic environment and reducing anoxic zone in the biofilters (Lin et al., 2015), withdrawing sediment in biofilters at regular intervals, and improving the biofilters performance and operation.

4.1.3. Effect of HLR on nitrogen transformations

Aquaponic systems were operated at HLR ranging from 0.10 to 2.5 m^3/m^2-day (Tables 4.2, 4.3, and 4.4). HLRs above 0.50 m^3/m^3-day did not affect nitrogen transformations in aquaponic systems. However, HLRs of 0.25 and 0.10 m^3/m^3-day negatively affected nitrite oxidation and TAN oxidation, respectively. Significant accumulations of nitrite and TAN concentrations were attributed to a decrease in nitrite and TAN oxidations, respectively.

Table 4.2. Summary of nitrogen and COD concentrations in the aquaponics operating at different HLRs in lettuce-based aquaponic systems.

DO in fish tanks (mg/L)	HLR (m^3/m^2-day)	DO in grow bed (mg/L)	TKN (mg N/L)	TAN (mg N/L)	NO_2^- (mg N/L)	NO_3^- accumulation rate (mg N/L-day)	COD (mg/L)
6.36 (0.21)	0.25	4.37 (0.40)[a]	8.9 (0.9)	0.59 (0.15)	0.62 (0.16)[a]	1.00 (0.17)	65.2 (4.5)
	1.5	5.93 (0.20)[b]	9.4 (0.8)	0.52 (0.16)	0.30 (0.05)[b]	0.90 (0.11)	58.6 (4.0)

(Note: Values are the mean of multiple data (n = 15) for TKN, TAN, NO_2^- and COD concentrations, n = 3 for NO_3^- accumulation rate, (n = 32) for DO concentrations, and values in parenthesis represent standard deviation. The superscripts a and b represent statistically different ($p < 0.05$) between two HLR levels.)

HLR of 0.25 m^3/m^2-day was found to significantly increase nitrite concentrations in lettuce-based aquaponics (Table 4.2). Average DO concentrations in the grow beds were 4.37 ± 0.40 mg/L (n = 32) and 5.93 ± 0.20 (n = 32) when operated at HLRs of 0.25 and 1.5 m^3/m^2-day,

respectively, supporting that a lower HLR level associated to a lower DO concentration in the biofilter and grow bed (Table 4.2). As the results, the decrease of HLR reduced nitrite oxidation rate, and denitrification promoted the accumulation of nitrite concentration at low DO level. Low HLR of 0.25 m^3/m^3-day meant slow recirculation rate, which limited the DO available for nitrite oxidation, but was not low enough to inhibit ammonia oxidation by ammonia oxidizing bacteria. To confirm the results, additional experiments using lettuce-based (Table 4.3) and chive-based aquaponic systems (Table 4.4) were operated at three HLRs of 0.10, 0.25, and 1.5 m^3/m^3-day. These experiments were designed to evaluate the effect of HLRs below 0.25 m^3/m^3-day on nitrite and ammonia oxidations.

Table 4.3. Summary of nitrogen and COD concentrations in the aquaponics operating at different HLRs in lettuce-based aquaponic systems.

DO in fish tanks (mg/L)	HLR (m^3/m^2-day)	DO in grow bed (mg/L)	TKN (mg N/L)	TAN (mg N/L)	NO_2^- (mg N/L)	NO_3^- accumulation rate (mg N/L-day)	COD (mg/L)
6.77 (0.15)	0.10	3.28 (0.61)[a]	8.7 (1.0)[a]	1.01 (0.19)[a]	0.60 (0.15)[a]	0.75 (0.06)[a]	74.2 (6.3)
	0.25	4.81 (0.48)[b]	8.7 (1.0)[a]	0.50 (0.03)[b]	0.35 (0.08)[b]	0.79 (0.07)[a]	77.2 (6.3)
	1.5	6.29 (0.15)[c]	8.9 (1.2)[a]	0.48 (0.07)[b]	0.20 (0.04)[c]	0.76 (0.03)[a]	74.8 (3.4)

(Note: Values are the mean of multiple data (n = 8) for TKN, TAN, NO_2^- and COD concentrations, n = 2 for NO_3^- accumulation rate, (n = 32) for DO concentrations, and values in parenthesis represent standard deviation. The superscripts a, b, and c represent statistically different ($p < 0.05$) between three HLR levels.)

HLR of 0.10 m^3/m^2-day negatively affected both TAN and nitrite oxidations, leading to an increase in TAN and nitrite concentrations (Tables 4.3 and 4.4). Thus, low HLR of 0.1 m^3/m^2-day limited the DO available for TAN and nitrite oxidations in aquaponic systems. HLR of 0.10 m^3/m^2-day also significantly caused the lowest DO concentrations in grow beds in both lettuce-

and chive-based aquaponic systems (Tables 4.3 and 4.4). Low HLR resulted in low DO concentrations; however, nitrite oxidation was more sensitive to DO than TAN oxidation because the nitrite accumulations were found at an HLR of 0.25 m^3/m^2-day while the TAN accumulations were found at 0.10 m^3/m^2-day. Moreover, it was apparent that the HLR of 0.5 to 2.5 m^3/m^2-day did not significantly affect the TKN, TAN and nitrite concentrations, and the accumulation of nitrate (Table 4.5). HLRs above 0.50 m^3/m^2-day also did not significantly improve TAN and nitrite oxidations. Therefore, the HLR of 1.5 m^3/m^2-day was high enough for maintaining good water quality for the floating-raft aquaponics in this study due to the efficient nitrification, disregarding the DO levels in fish tanks and biofilters.

Table 4.4. Summary of nitrogen and COD concentrations in the aquaponics operating at different HLRs in chive-based aquaponic systems.

DO in fish tanks (mg/L)	**HLR (m^3/m^2-day)**	**DO in grow bed (mg/L)**	**TKN (mg N/L)**	**TAN (mg N/L)**	**NO_2^- (mg N/L)**	**NO_3^- accumulation rate (mg N/L-day)**	**COD (mg/L)**
6.98 (0.27)	0.10	2.76 (0.68)[a]	10.9 (2.1)[a]	1.15 (0.34)[a]	0.70 (0.17)a	1.56 (0.17)[a]	83.7 (6.1)
	0.25	3.93 (0.65)[b]	9.9 (0.9)[a]	0.65 (0.15)[b]	0.39 (0.08)[b]	1.39 (0.11)[a]	79.2 (3.9)
	1.5	6.07 (0.27)[c]	10.5 (1.8)[a]	0.56 (0.14)[b]	0.20 (0.07)[c]	1.36 (0.04)[a]	78.5 (8.3)

(Note: Values are the mean of multiple data (n = 18) for TKN, TAN, NO_2^- and COD concentrations, n = 2 for NO_3^- accumulation rate, (n = 70) for DO concentrations, and values in parenthesis represent standard deviation. The superscripts a, b, and c represent statistically different ($p < 0.05$) between three HLR levels.)

Table 4.5. Summary of nitrogen and COD concentrations in the aquaponics operating at different HLRs.

Plant types	Run no.	DO in fish tanks (mg/L)	HLR (m^3/m^2-day)	TKN (mg N/L)	TAN (mg N/L)	NO_2^- (mg N/L)	NO_3^- accumulation rate (mg N/L-day)	COD (mg/L)
Pak choi	1	6.07 (0.26)	1.0	3.5 (1.2)	1.24 (0.73)	0.44 (0.18)	0.56 (0.20)	66.3 (6.0)
			1.5	4.2 (1.0)	1.91 (0.58)	0.52 (0.15)	0.50 (0.09)	73.3 (6.6)
	2	5.88 (0.26)	2.0	4.5 (1.5)	1.71 (0.69)	0.53 (0.12)	0.45 (0.17)	77.5 (7.0)
			2.5	4.2 (1.5)	2.01 (0.99)	0.43 (0.17)	0.33 (0.09)	78.0 (12.2)
	3	6.72 (0.35)	2.0	6.7 (0.8)	1.05 (0.27)	0.39 (0.17)	0.52 (0.13)	72.8 (10.4)
			1.5	6.5 (0.7)	0.98 (0.26)	0.43 (0.16)	0.63 (0.25)	74.6 (10.7)
Lettuce	4	6.39 (0.34)	1.0	10.6 (2.7)	1.10 (0.29)	0.26 (0.09)	0.72 (0.13)	91.3 (6.4)
			1.5	8.2 (2.9)	0.69 (0.37)	0.16 (0.06)	0.63 (0.07)	89.0 (8.5)
	5	6.60 (0.32)	0.5	11.5 (0.9)	0.89 (0.10)	0.28 (0.05)	0.90 (0.06)	64.2 (4.2)
			1.5	10.3 (1.4)	0.69 (0.09)	0.23 (0.06)	0.90 (0.08)	61.6 (4.3)

(Note: Values are the mean of multiple data (n = 15 for TKN, TAN, NO_2^- and COD concentrations, n = 3 for NO_3^- accumulation rate, n = 37 for DO from pak-choi based-, and n = 32 from lettuce based-aquaponics). No significant difference on nitrogen concentrations ($p < 0.05$) between two HLR levels in each run. The aquaponic systems were operated at feeding rates of 50 g feed/day (Run. No. 1-3) and 35 g feed/day (Run no. 4-5).

Further research is needed to evaluate the effects of other operational parameters on organic nitrogen transformations in the recirculating water. However, it should be noted that comparison of nitrogen concentrations among different runs was not performed in this section due to seasonal effect, which caused fluctuations in nitrogen concentrations (Ge et al., 2016). This range of HLR was also comparable to other types of aquaponic system. For example, Endut et al. (2010) reported that the HLR of 1.28 m^3/m^2-day was optimum for nitrification, fish production, and plant growth. It is important to note that NOB are more susceptible to DO level in the system due to their lower affinity for DO than the AOB (Ge et al., 2015; Semerci and Hasilci, 2016; Wang et al., 2014). Ammonia oxidizing archaea (AOA) were unlikely in this aquaponic system since AOA are not competitive for oxidizing ammonia under high ammonium concentrations (Brown et al., 2013; Martens-Habbena et al., 2009; Schmautz et al., 2017). The results suggest that nitrite concentration could be an indicator of HLR levels sufficient for maintaining effective nitrification and minimizing denitrification in an aquaponic system. Thus, HLR could be used as an important operating parameter in designing and operating an effective aquaponic system.

4.1.4. Effect of pH on nitrogen transformations

TAN concentrations at acidic pH levels (6.0 ± 0.2 and 5.2 ± 0.2) were higher than TAN concentrations at near neutral pH level (7.1 ± 0.4 mg/L (phase I), and 6.9 ± 0.3 mg/L (phase II)) in chive-based aquaponic systems (Figure 4.6a). Average TAN concentrations at steady state at pH of 6.0 (2.6 ± 0.6 mg N/L) were significantly higher (p = 0.001) than those at pH 7.0 (0.8 ± 0.2 mg N/L) (Figure 4.6a and Table 4.7). At pH of 6.0, average TAN concentrations at steady state increased significantly up to 2.6 mg N/L (Figure 4.6 and Table 4.7). These results showed that in spite of decreased ammonia oxidation rate, the AOB in aquaponic systems were able to tolerate low pH of 6.0 (Gieseke et al., 2006). At such low pH, higher biofilter surface area is needed to reduce the TAN concentration to that of TAN concentration at pH of 7.0 (Timmons et al., 2002). Growing plants that produce higher total root surface area (e.g., tomato) for rhizobacteria in combination with other plants is recommended to maintain low TAN concentration in the aquaponic system when the pH drops to 6.0 (see Table 4.6 and Figure 4.5 for root surface areas and root morphologies, respectively, of the four plants grown in the aquaponic systems). Nevertheless, at pH of 5.2, TAN concentrations continued to increase (r^2 = 0.989, p <0.001) and

accumulated in the recirculating water. TAN-to-nitrite ratio varied from 2.4 to 6.6 when the pH was maintained at neutral level (7.0 ± 0.4). However, the ratio drastically increased to nearly 20 and 100 when the pH decreased to 6.0 and 5.2, respectively, suggesting the extreme sensitivity of AOB to low pH levels in the aquaponic system.

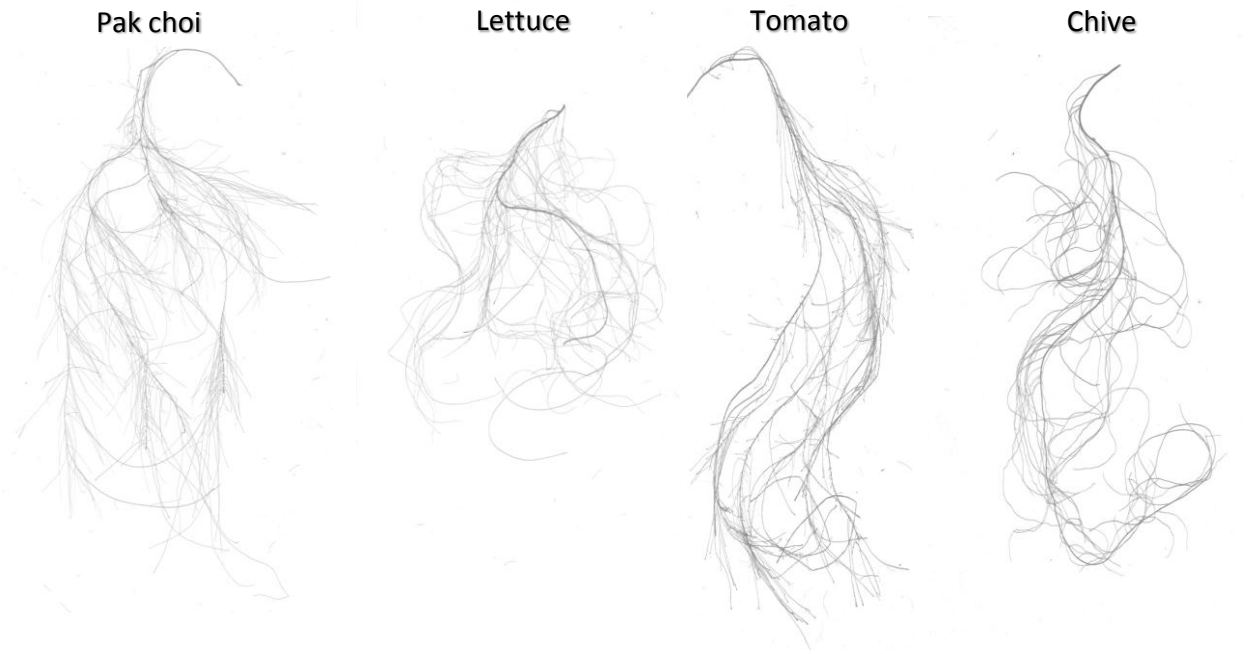

Figure 4.5. Morphologies of plant roots (pak choi. lettuce, tomato, and chive) grown in the floating-raft aquaponic systems

Table 4.6. Root surface areas of plants (pak choi, lettuce, tomato, and chive) grown in the floating-raft aquaponic systems

Root surface area	Pak choi	Lettuce	Tomato	Chive
cm^2/g (root dry wt.)	2,607 (546)	2,016 (278)	481 (210)	800 (331)
cm^2/plant	724 (251)	474 (109)	6.01×10^4 (1.71×10^4)	227 (104)

(Note: Values are the mean of multiple data (n = 24 for pak choi, lettuce, and chive, n = 12 for tomato). Values in () represents standard deviation.)

Table 4.7. Operational parameters (DO and pH levels) and system performances (nitrogen and COD concentrations, and NO_3^- accumulation rates) in chive-based aquaponic systems at different pH levels

Phase no.	pH levels	DO in fish tank (mg/L)	pH	TKN (mgN/L)	TAN (mgN/L)	NO_2^- (mgN/L)	NO_3^- accumulation rate (mgN/L/d)*	Range of NO_3^- (initial - final) (mg N/L)	COD (mg/L)
I	pH 7.0	6.9 (0.4)	7.1 (0.2)[a]	7.8 (1.3)	0.8 (0.2)[a]	0.21 (0.03)[a]	1.75	143 - 187	46.8 (3.1)
	pH 6.0	6.8 (0.4)	6.0 (0.2)[b]	8.5 (1.8)	2.6 (0.6)[b]	0.13 (0.04)[b]	1.33	177 - 218	65.1 (5.6)
II	pH 7.0	6.9 (0.3)	6.9 (0.3)[a]	8.0 (2.1)	0.8 (0.1)[a]	0.21 (0.06)[a]	1.52	178 - 230	53.8 (6.0)
	pH 5.2	7.2 (0.4)	5.2 (0.2)[c]	10.1 (2.4)	6.6 (5.2)[c,**]	0.07 (0.03)[c]	0.79	143 - 168	49.1 (3.1)

(Note: Values are the mean of multiple data (n = 15) for TKN, TAN, NO_2^- and COD concentrations, (n = 3) for NO_3^- accumulation rate, (n = 29 (phase I), n=32 (phase II)) for pH and DO concentrations, and values in parenthesis represent standard deviation. The superscripts a, b, and c represent statistically different ($p < 0.05$) between three pH levels. * NO_3^- accumulation rates were shown instead of the average values because NO_3^- concentrations did not reach steady state. ** TAN accumulated over time at pH of 5.2.)

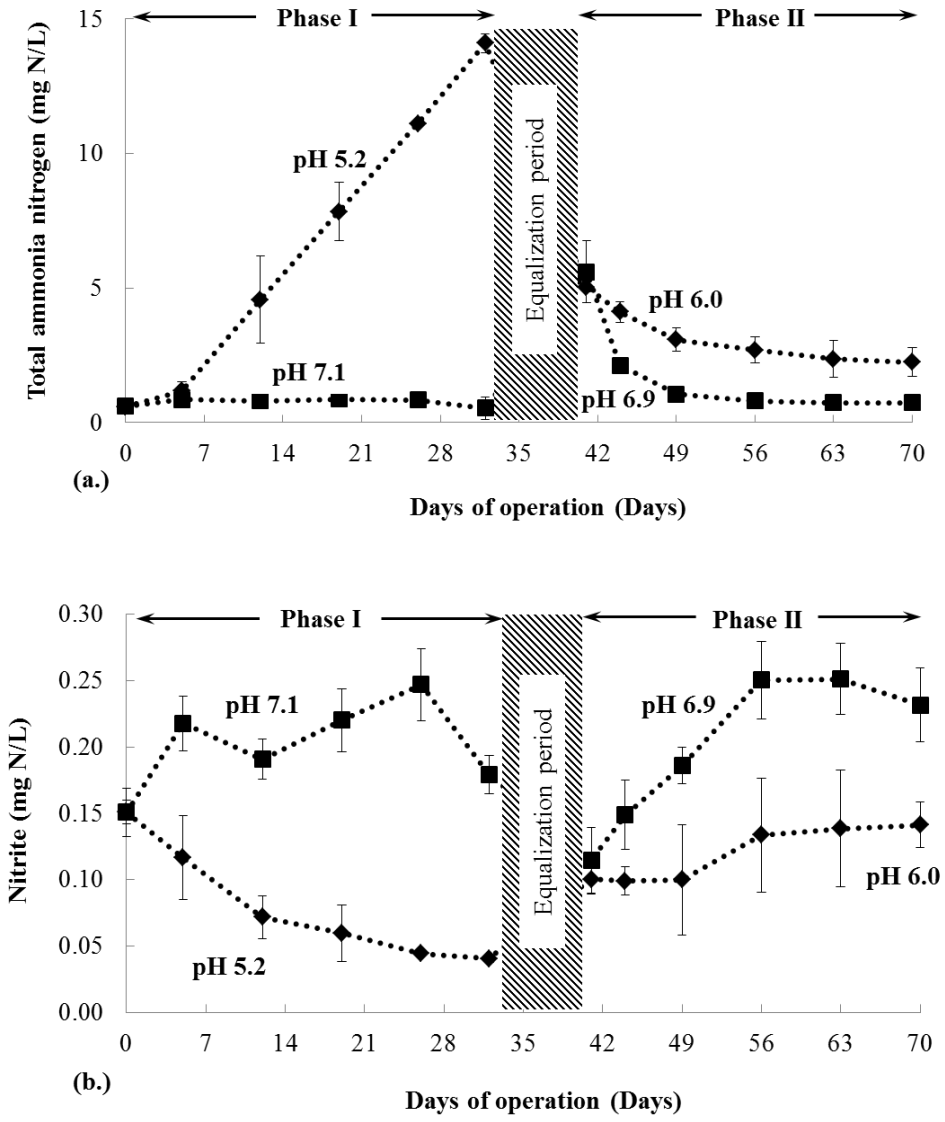

Figure 4.6. TAN (a.) and nitrite (b.) concentrations in chive-based aquaponic systems at pH of 5.2, 6.0, and 7.0. An error bar represents the standard deviation of TAN and nitrite concentrations from three aquaponic systems operating at the same pH condition.

Interestingly, nitrite concentrations in the aquaponic systems operating at the two low pH levels were significantly lower than the aquaponics operating at pH of 7.0 (p <0.001) (Table 4.7 and Figure 4.6). Nitrite oxidation efficiency increased when TAN oxidation efficiency decreased under low pH conditions, as evident from low nitrite concentration. The decrease in nitrite concentrations was attributed to lower nitrite generation by AOB at low pH levels, leading to a

lower nitrite concentration. Nitrite concentrations dropped significantly by 35% and 65% when the pH levels decreased due to nitrification from 7.0 to 6.0, and 7.0 to 5.2, respectively. Nitrite concentration, however, decreased only by 1.5-fold whereas TAN concentration increased by 3.5-fold when the pH decreased from 7.0 to 6.0 due to nitrification (Figure 4.6), suggesting that the low pH levels negatively affected both ammonia and nitrite oxidation rates. To confirm that pH affected TAN and nitrite concentrations, the author compared TAN and nitrite concentrations in lettuce-based aquaponic systems operating at two different pH levels (6.1 and 6.9) (Table 4.8). Significant differences on TAN (p=0.001) and nitrite (p=0.002) concentrations were found at the two pH levels (Table 4.8). Results agreed to the effect of pH on nitrogen transformations in chive-based aquaponic systems.

Thus, the decrease in nitrite concentrations was attributed to lower nitrite generation by AOB at low pH levels, leading to a lower nitrite concentration. Plants can take up ammonium, nitrite and nitrate nitrogen from recirculating water, preferably at a slightly acidic range (pH of 5.0-6.0) (Resh, 2013). Thus, aquaponic systems can be operated at acidic pH levels as long as TAN concentration does not accumulate perpetually, and free ammonia and nitrite concentrations do not reach a toxicity level for fish (<1.6 mg N/L of unionized ammonia (NH_3) and <8.2 mg N/L of NO_2^-) (T. Popma and Masser, 1999).

Table 4.8. Operational parameters (DO and pH levels) and system performances (nitrogen and COD concentrations, and NO_3^- accumulation rates) in lettuce-based aquaponic systems at two pH levels (6.1 and 6.9)

DO in fish tank (mg/L)	pH	TKN (mg N/L)	TAN (mg N/L)	NO_2^- (mg N/L)	NO_3^- accumulation rate (mg N/L-day)	COD (mg/L)
6.5 (0.4)	6.1 (0.3)	8.2 (1.0)	2.2 (0.6)[a]	0.15 (0.04)[a]	0.64 (0.11)	85.8 (3.9)
6.3 (0.3)	6.9 (0.3)	8.2 (1.2)	0.7 (0.3)[b]	0.26 (0.08)[b]	0.68 (0.07)	84.5 (6.8)

(Note: Values are the mean of multiple data (n = 15) for TKN, TAN, NO_2^- and COD concentrations, (n = 3) for NO_3^- accumulation rate, n=32 for pH and DO concentrations, and values in parenthesis represent standard deviation. The superscripts a and b represent statistically different ($p < 0.05$) between two pH levels.

4.1.5. Nitrogen transformations in aquaponic systems at optimum DO, HLR, and pH in different plant based aquaponic systems

At pH range of 6.8-7.0 and a constant HLR of 1.5 m^3/m^2/day, TKN, TAN, and nitrite concentrations showed some variation. However, no accumulations or depletions of those nitrogen species were observed ($p > 0.05$) in each harvesting cycle (Table 4.9). Ammonia and nitrite concentrations reached a steady state at this pH range. Sufficient DO levels (6.8-7.4 mg/L, Table 4.9) associated with high HLR of 1.5 m^3/m^2/day facilitated effective nitrification throughout the biofilters and the grow beds (Wongkiew et al., 2017b).

Table 4.9. Operational parameters (DO and pH levels) and system performances (nitrogen and COD concentrations, and NO_3^- accumulation rates) in pak choi-, lettuce-, tomato-, and chive-based aquaponic systems

Plant types	DO in fish tank (mg/L)	pH	TKN (mgN/L)	TAN (mgN/L)	NO_2^- (mgN/L)	NO_3^- accumulation rate (mgN/L/d)*	Range of NO_3^- (initial - final) (mg N/L)	COD (mg/L)
Pak choi	7.4 (0.3)	6.8 (0.2)	4.2 (1.4)	1.1 (0.2)	0.25 (0.07)	0.82	150 - 180	76.3 (5.5)
Lettuce	6.9 (0.2)	6.9 (0.1)	8.0 (1.5)	0.7 (0.1)	0.24 (0.06)	0.57	211 - 229	56.1 (2.9)
Tomato (12 plants)	6.8 (0.3)	7.0 (0.2)	7.8 (1.4)	0.9 (0.1)	0.15 (0.05)	$6.9(10^{-4})t^2$ - 0.11t+ 2.4	139 - 60	69.2 (6.9)
Tomato (6 plants)	6.4 (0.4)	6.8 (0.2)	8.3 (0.9)	0.9 (0.1)	0.24 (0.04)	$11.4(10^{-4})t^2$ – 0.13t + 3.4	138 - 163	84.5 (6.7)
Chive	6.9 (0.2)	7.0 (0.4)	7.9 (1.7)	0.8 (0.2)	0.20 (0.05)	1.63	143 - 249	50.3 (5.9)

(Note: Values are the mean of multiple data for TKN, TAN, NO_2^- and COD concentrations (n = 15 for pak choi and lettuce, n= 36 for tomato, n= 30 for chive), NO_3^- accumulation rate (n = 3), DO concentrations (n = 37 for pak choi, n = 32 for lettuce, n= 90 for tomato, n= 61 for chive). Values in parenthesis represent standard deviation. * NO_3^- accumulation rates were shown instead of the average values because NO_3^- concentrations did not reach steady state.

Higher TAN concentrations at steady state relative to nitrite concentrations (p <0.001), irrespective of plant species, suggested that other biochemical processes such as degradation of organic nitrogen (TKN in Table 4.9), urea excretion (Timmons et al., 2002), and nitrite reduction at anoxic conditions in the upflow biofilter (Wongkiew et al., 2017b) could take place with nitrite and ammonia oxidations in the aquaponic systems, leading to the difference in concentrations. However, nitrate concentrations in pak choi, lettuce and chive-based aquaponic systems increased over time and followed a zero-order kinetics (r^2 = 0.923, 0.965, and 0.973, p = 0.002, <0.001, and <0.001, respectively), suggesting the imbalance of nitrate generation via nitrification, nitrate uptake by plants, and nitrogen loss via denitrification (Wongkiew et al., 2017a, 2017b). In contrast, the nitrate accumulation rate in recirculating water of tomato-based aquaponic systems best-fitted the mixed-order (r^2 = 0.980 (12 plants), r^2 = 0.822 (6 plants), p <0.001, Table 4.9), showing nitrate depletion when nitrate uptake rate by plants exceeded the nitrogen input from the feed (Wongkiew et al., 2017a, 2017b).

4.2. Monitor the transformations of different forms of nitrogen in an aquaponic system under different conditions

Part 1: Nitrogen products distributions in aquaponic systems

4.2.1. Transformations of different forms of nitrogen in different plant-based aquaponic systems

Fish feed was the major nitrogen input to the aquaponic systems. Nitrogen fixation was negligible in this study because non-legume plants were used in this study (no legume forming on plant roots, see Figure 4.5). No microalgae and nitrogen-fixing bacteria growth in the aquaponic systems existed. (Biofilters and fish tanks were covered from sunlight. Less than 1% relative abundance of *Cyanobacteria*, excluding root chloroplast, was found in all samples (Figure C.5)). Plant biomass, fish biomass, sediment, accumulated nitrate (including accumulated ammonium at pH of 5.2) in recirculating water, and nitrogen gas were the major nitrogen outputs (Figure 4.7). Fish excreted TAN from excess L-amino acids derived from fish feed protein via transamination and deamination in fish liver (Wongkiew et al., 2017a, 2017b). Then the excreted TAN was oxidized to nitrite and nitrate, contributing to nitrate in aquaponic systems (N accumulated in water, Figure 4.7). Nitrate was a major source of nitrogen for plant

assimilation and nitrogen loss via denitrification due to the high concentrations of nitrate in the aquaponic systems (Table 4.9). Tomato (12 plants) showed the highest plant NUE (49.2%), followed by tomato (6 plants, NUE = 32.1%), pak choi (23.2%), lettuce (13.7%), and chive (1.5%), respectively. Figure 4.8 shows that percent nitrogen recovery in pak choi was not different from percent nitrogen recovery lettuce. Nitrogen in fish biomass varied from 26.3 to 35.5% (Figure 4.8).

Nitrate accumulation in the water was transformed into plant biomass via assimilation and nitrogen gas via denitrification (Erguder et al., 2008; Öhlund and Näsholm, 2002; Wongkiew et al., 2017b; Zhao et al., 2015). Among the four plant species, the highest nitrate accumulation rate in recirculating water (Table 4.9) occurred in chive-based aquaponic system (pH 7.0), and the rapid nitrate depletion occurred in the tomato-based aquaponic system due to the high biomass yield of tomato. This nitrate depletion indicated that nitrate uptake by tomato was higher than the nitrogen input from the fish feed, and tomato withdrew the resource of nitrate from aquaponic systems.

Inevitable nitrogen loss, due to denitrifying heterotrophic bacteria utilizing organic carbon (Erguder et al., 2008; Vlaeminck et al., 2009; Wongkiew et al., 2017b; Zhao et al., 2015) (presented as COD, Table 4.9). However, nitrogen losses from the four aquaponic systems were in the same range of nitrogen loss (19.2-25.5%) from aquaponic systems with no plants (21.1 ± 3.7 %, n = 3), suggesting that plants did not decreased nitrogen loss via denitrification in aquaponic systems. Major compositions of the sediment could be a mixture of fish feces and heterotrophic and autotrophic cells (Wongkiew et al., 2017b). Anoxic condition could occur in the first biofilter, leading to nitrogen loss, degradation of organic compounds, and production of dissolved organic matter in the aquaponic biofilter (Wongkiew et al., 2017a, 2017b). However, nitrogen output in sediment was relatively low compared to the other outputs, suggesting that nitrogen recovery from the sediment withdrawn from biofilters may not have significant economic contribution. Therefore, increasing nitrate uptake rate (e.g., increasing number of rapid growing plants), balancing nitrification using an optimum feed-to-plant ratio, and maintaining a stable nitrate concentration are some of the strategies to achieve the highest NUE of aquaponic systems.

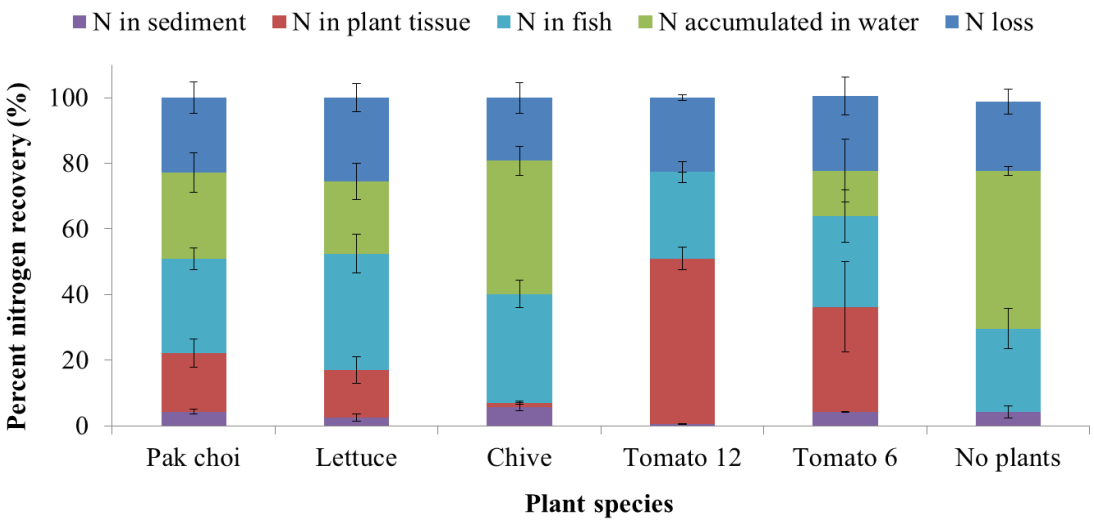

Figure 4.7. Nitrogen distribution of products in aquaponic systems operating at HLR of 1.5 m^3/m^2-day. Error bars represent the standard deviations of aquaponics operated in triplicate.

4.2.2. Transformations of different forms of nitrogen at different feeding rates

At feeding rates ranging from 15 to 50 g/day, nitrogen from the fish feed in the aquaponic systems transformed into five major products, namely nitrogen in fish, nitrate accumulation in recirculating water, nitrogen in plant biomass, and nitrogen in the sediment withdrawn from the biofilters (Figure. 4.8). The nitrate accumulation in aquaponic systems indicated that nitrate generation rates were higher than the plant requirement. The nitrate accumulation could be used as an indicator of the balance between the rate of fish feeding and vegetable yield in aquaponic systems. Figure 4.8 shows that reducing feeding rate when nitrate accumulation occurred, reduced nitrogen loss in aquaponic systems.

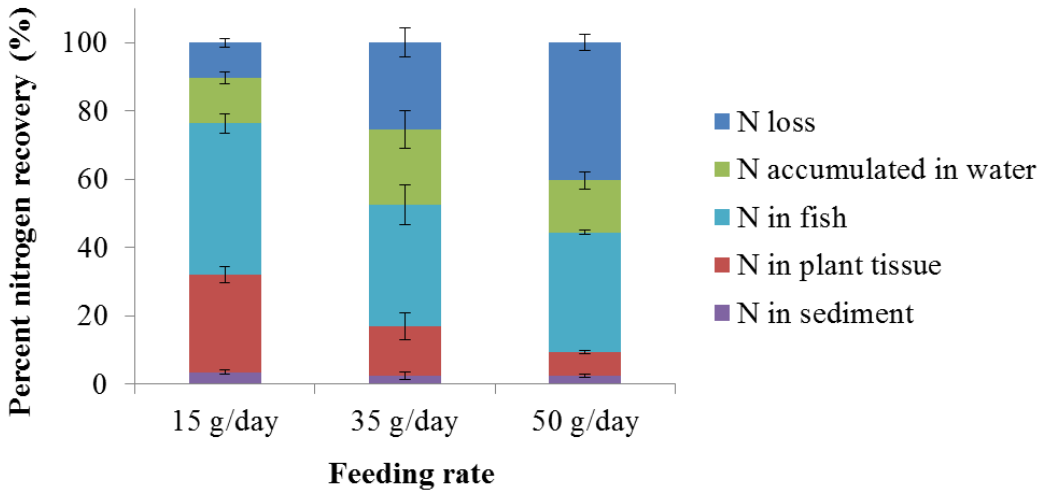

Figure 4.8. Nitrogen distribution of products in lettuce-based aquaponic systems operating at feeding rate of 15 g/day (n = 3), 35 g/day (n = 6), and 50 g/day (n = 3)

Percent nitrogen loss in lettuce-based aquaponics accounted for 40.4 % when the fish feed was 50 g/day, but the nitrogen loss decreased to 25.5 % and 10.3% when feeding rates were reduced to 35 and 15 g/day, respectively. Nitrogen in lettuce biomass at feeding rates of 15 to 50 g/day (7.5-8.3 gN/harvesting cycle) were not significantly different because nitrate concentrations in both conditions exceeded plant requirements. Significant difference on fish biomass yields was found between feeding rate of 15 and 35 g/day ($p<0.001$), but not significant different between feeding rates of 35 and 50 g/day ($p = 0.271$) due to the constant feeding rates and feeding frequency that limited exponential fish growth for adult fish (Ng et al., 2000). Although sediment concentrations in biofilters between the three feeding conditions were not significantly different ($p>0.05$), TKN and COD concentrations suggested that organic matter in the biofilters could be hydrolyzed and decayed into soluble organic forms as a carbon source for heterotrophs that contributed to denitrification (Li et al., 2016; C. Y. Wang et al., 2016). The effect of feeding rate on nitrogen loss, which resulted from denitrification, could be due to the low DO level associated with the accumulation of feces and sediment in biofilters in which high microbial activities. Future research in optimizing feeding rate and maximizing nitrogen uptake by plants to minimize nitrogen loss, by maintaining the balance between nitrate accumulation and nitrate uptake, is recommended.

4.2.3. Transformations of different forms of nitrogen at different HLRs (feeding rate 35 g/day and 50 g/day)

At feeding rate of 35 g/day, each nitrogen product in aquaponic systems operating at HLRs ranging from 0.25 to 1.5 m^3/m^2-day varied within a certain range (Figure 4.9). Percent nitrogen loss varied from 25.7 to 30.5% while percent nitrogen in plant biomass (lettuce) varied from 13.1 to 16.6% (Figure 4.9). Nevertheless, no difference in nitrogen loss was observed at HLR of 0.25 to 1.5 m^3/m^2-day. Compared to aquaponics at feeding rate of 35 g/day, higher nitrogen losses ranging from 43.6 to 47.3 % were found at feeding rate of 50 g/day although aquaponic systems were operated at high HLRs ranging from 1.0 to 2.5 m^3/m^2-day and high DO levels (Figure 4.10). Percent nitrogen recovery in pak choi biomass (19.4-22.3 %) was higher than lettuce. However, HLRs ranging from 1.0 to 2.5 m^3/m^2-day in the pak choi-based aquaponic systems did not apparently affect NUE and nitrogen loss.

The results suggested that operating aquaponics at high HLR or maintaining high DO level in the fish tank was less efficient, in term of reducing nitrogen loss, than reducing the feeding rate. This range of HLRs at the two feeding rates and plant species did not increase NUE. The HLRs levels did not have a significant effect on nitrogen loss and denitrification even if HLR could increase oxygen transfer in the biofilters and aquaponic grow bed. HLR did not efficiently improve NUE in aquaponic systems, and increasing HLR was not a convincing strategy to increase nitrogen recovery in aquaponic systems. However, as discussed earlier, HLR was important to maintain high DO level for efficient TAN and nitrite oxidations and prevent the accumulations of TAN and nitrite. Results also suggested that HLR can be maintained as low as 0.25 m^3/m^2-day if oxygen transfer rate is high enough to prevent TAN and nitrite accumulations that reach toxic threshold levels and are toxic to fish (Thomas Popma and Masser, 1999; Wongkiew et al., 2017b). Therefore, HLR did not have a huge effect on nitrogen products distributions, but it was important to transfer oxygen for a high DO concentration and maintain a good nitrification.

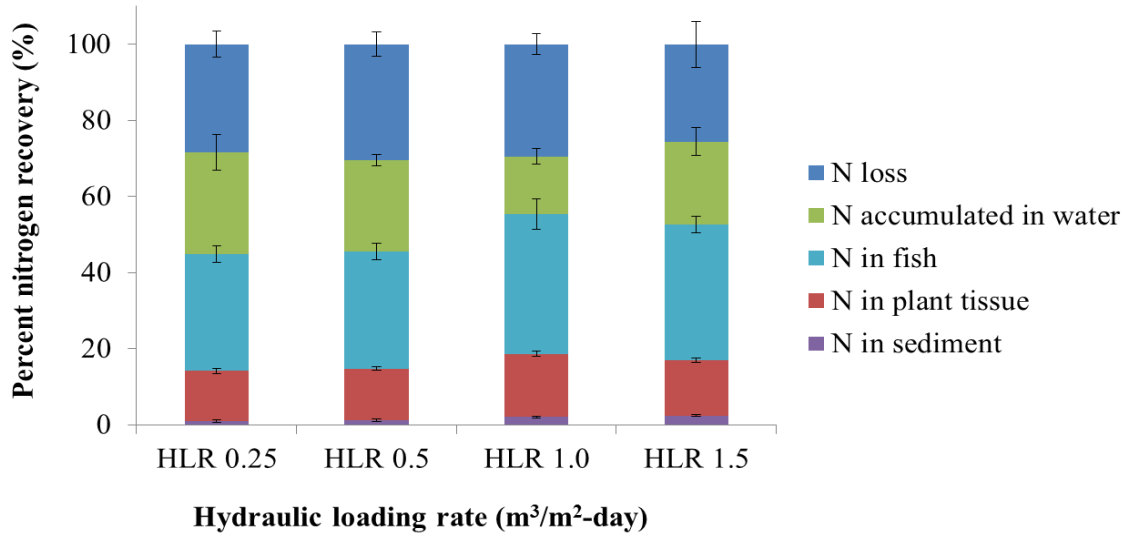

Figure 4.9. Nitrogen distribution of products in aquaponic systems operating at different HLRs and a constant feeding rate of 35 g/day. Error bars represent the standard deviations of aquaponics operated in triplicate.

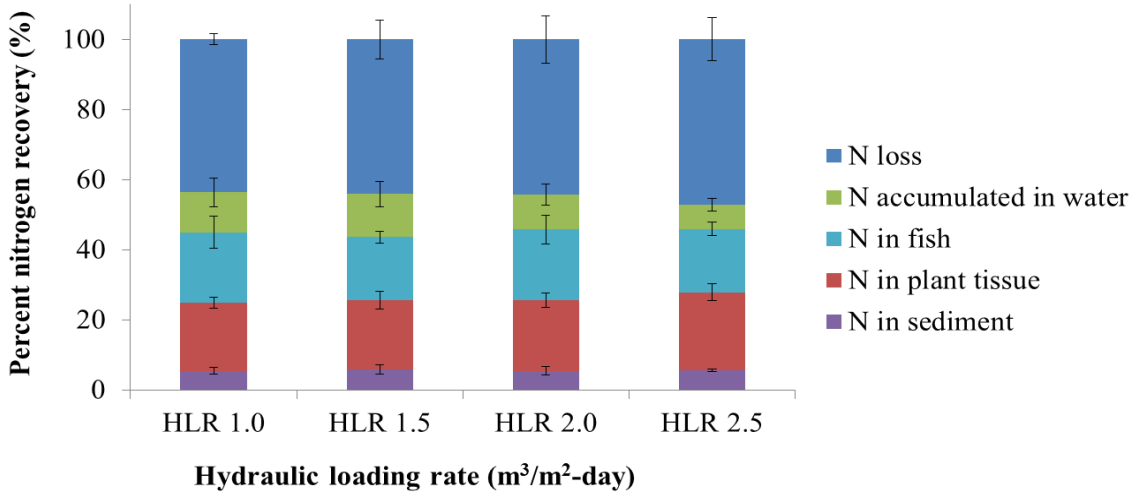

Figure 4.10. Nitrogen distribution of products in aquaponic systems operating at different HLRs and a constant feeding rate of 50 g/day. Error bars represent the standard deviations of aquaponics operated in triplicate.

4.2.4. Transformations of different forms of nitrogen at different DO levels

DO levels did not have a significant effect on nitrogen products distribution in aquaponic systems although the study in previous sections showed that DO affected nitrogen loss and denitrification in aquaponic systems. In this section, nitrogen losses at low DO (3.97 mg/L) and high DO (7.44 mg/L) levels were not found to be significantly different (p=0.777). The reasons could be due to the interference of plants that assimilated nitrate from the recirculating water. Thus, as discussed previously, the author compared the effect of DO on nitrogen loss in aquaponic systems with no plants (a higher accuracy test with natural abundance nitrogen isotopic compositions) in sections 4.1.1 and 4.1.2.

Nitrogen uptake by fish at low DO and high DO levels were not significantly different (p=0.539) (Figure 4.11), suggesting that tilapia can thrive and be productive when DO concentrations in fish tank were higher than 3.97 mg/L (Thomas Popma and Masser, 1999). Similarly, pak choi at the low DO level can assimilate nitrate nitrogen as effectively as pak choi at the high DO level (p=0.612) (Figure 4.11), suggesting that growing pak choi is recommended in aquaponic systems at low DO levels (Zou et al., 2016a). It is important to note that denitrification was unavoidable under all conditions. Low denitrification rate occurred even under high DO levels or occurred at micro-aerobic zones where oxygen transfer was limited (e.g., stagnant zone, inner layer of biofilm, and anoxic sediment zone) (Lin et al., 2015; Yin et al., 2015). Only maintaining high DO levels was not a convincing strategy to increase NUE in aquaponic systems. Integrated strategy such as balancing feed-to-plant ratio at high DO levels could be another approach to maximizing nitrogen recovery in aquaponic systems.

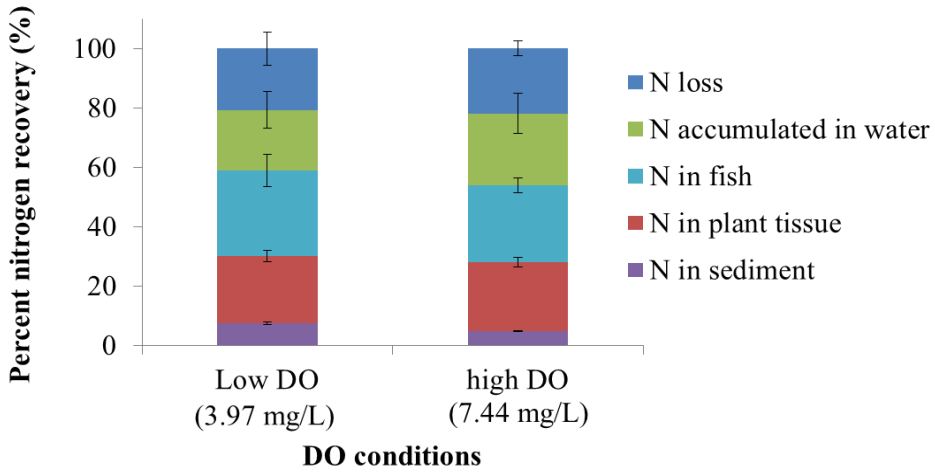

Figure 4.11. Nitrogen distribution of products in pak choi-based aquaponic systems operating at different DO levels and a constant feeding rate of 35 g/day. Error bars represent the standard deviations of aquaponics operated in triplicate.

Part 2: Nitrogen isotope studies in aquaponic systems

4.2.5. Evaluation of natural abundance nitrogen isotopic composition ($\delta^{15}N$) values

All nitrogen outputs showed enrichments of ^{15}N relative to the fish feed (input) (Figure 4.12). The ^{15}N enrichments in the products could be attributed to kinetic isotope effects (KIE) associated with nitrogen assimilation (deamination and transamination) in the fish, microbial transformations of nitrogen species, and assimilatory nitrogen uptake by plants (Lam et al., 2015; C. Y. Wang et al., 2016). As expected from normal KIE, ^{15}N accumulated in fish muscle tissue and solid excreta (G. Chen et al., 2012; Peterson and Fry, 1987; Xia et al., 2013). The $\delta^{15}N$ values of plant tissues increased from roots to leaves and fruits (tomato) (Figure 4.11) due to isotope fractionation associated with nitrate translocation and assimilatory nitrate reduction in roots, stems, leaves, and fruits (tomato) (section 4.2.6). Chive did not have stems, and all part above chive roots was defined as chive leaves in this study. The $\delta^{15}N$ values in stems, leaves, and fruits (tomato) were lower than the $\delta^{15}N$ values of nitrate extracted from stems and leaves, indicating that pak choi, lettuce, chive, and tomato assimilated nitrate to the plant cells. Since efflux from the roots occurred in the aquaponic systems, $\delta^{15}N$ values of nitrate in plant cells also resulted in the enrichment of ^{15}N in recirculating water. However, the $\delta^{15}N$ values of all outputs

could not close the nitrogen isotope mass balance (Eq. 3.5) because all of the major nitrogen measured (products) have higher $\delta^{15}N$ values compared to feed, indicating loss of ^{14}N from the system. Thus, ^{15}N depleted N_2 gas, which would be generated via denitrification in the aquaponic systems, was believed to be the unmeasured isotopic output that was missing in the isotopic mass balance.

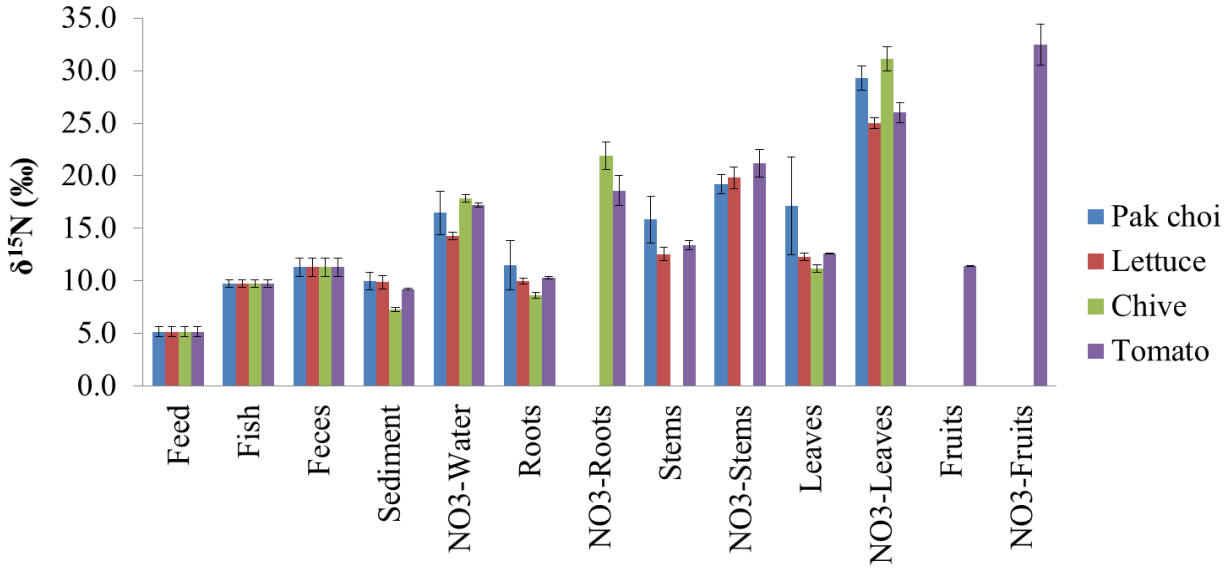

Figure 4.12. Natural abundance $\delta^{15}N$ values of bulk nitrogen and nitrate (NO_3^-) in feed (precursor) and products in aquaponic systems operating at high DO levels, feeding rate of 35 g feed/ day, and HLR of 1.5 m^3/m^2-day

In this study, N_2 depleted in ^{15}N relative to the fish feed was most likely generated in low oxygen regions of the biofilters where there was high metabolic activity of denitrifiers (J. P. Blancheton et al., 2013; Lu et al., 2014a; Michaud et al., 2006; Rathnayake et al., 2015) and led to high $\delta^{15}N$ values of nitrate in the recirculating water (Figure 4.12). The increase in $\delta^{15}N$ values of nitrate was due to isotope fractionation associated with denitrification (Casciotti and Buchwald, 2012; Dähnke and Thamdrup, 2013; Vavilin and Rytov, 2015). The enrichment in ^{15}N of residual nitrate in the recirculating water due to denitrification process has also been reported in other biogeochemical and laboratory scale studies, supporting the nitrogen gas generation from the aquaponic systems (Frey et al., 2014; Vavilin and Rytov, 2015). Other biochemical processes related to the enrichment of ^{15}N, such as ammonia volatilization (Lee et

al., 2011; Nômmik et al., 1994) and nitrifier denitrification (Casciotti and Buchwald, 2012) were negligible in this study due to low TAN concentration (< 2 mg N/L) and pH of 6.7-7.0, which would be less favorable for ammonia volatilization (Rochette et al., 2013).

4.2.6. Nitrate reduction in plants

$\delta^{15}N$ values in recirculating water, $\delta^{15}N$ values of roots, stems, leaves, fruits (tomato) and nitrate in stems, leaves, and fruits (tomato), indicates nitrogen isotope fractionation associated with incomplete nitrate assimilation into plant organic nitrogen (Figure 4.12). Isotope mass balance dictates no net isotope fractionation would be expected if assimilatory nitrate reduction in the pak choi, lettuce, chive, and tomato were complete. Previous studies showed residual nitrate in stems and leaves is enriched in ^{15}N relative to ambient nitrate due to assimilation by plants (Evans et al., 1996; Mariotti et al., 1982; Tcherkez and Farquhar, 2006). Incomplete assimilatory nitrate reduction in the plants would facilitate the efflux of nitrate with high $\delta^{15}N$ values from the plant root to the recirculating water (Kolb and Evans, 2003). Thus, in the aquaponic systems, high $\delta^{15}N$ values of nitrate in the recirculating water may be due in part to root efflux of nitrate, which decreased the nitrate reduction efficiency and NUE.

The assimilatory nitrate reduction efficiencies (F_3/F_1) was calculated according to Evans (2001), and Kolb and Evans (2003) (ε due to nitrate reductase (ε_f) = 17‰, $\varepsilon_p = \varepsilon_f - \varepsilon_f(F_3/F_1)$, ε_p = measured actual isotope effect, F_3 is assimilatory flux and F_1 is uptake flux). Nitrate reduction efficiency from recirculating water in pak choi stems (84 ± 14%) was higher than leaves (25 ± 14%), and the other plants also showed similar results (Table 4.10). The results suggested that assimilatory nitrate reduction could occur in pak choi, lettuce,chive, and tomato stems by nitrate translocation from roots, despite small amount of inorganic assimilation reported in stems (Kalcsits et al., 2014; Kalcsits and Guy, 2013). Higher assimilatory nitrate reduction efficiency could be achieved by balancing between nitrate input and nitrate uptake by plants, and avoiding environmental stress to the plants (Lam et al., 2015; Wongkiew et al., 2017a). However, the fact that $\delta^{15}N$ values of nitrate in the recirculating water increased even in the absence of plants (section 4.1.2) indicates a process affecting the isotopic composition of nitrogen other than incomplete plant assimilation of nitrate.

Table 4.10. Nitrate (NO_3^-) reduction efficiency (%) from recirculating water of four plants in floating-raft aquaponic systems

	Pak choi	Lettuce	Chive	Tomato
Stems	84 (14) %	67 (6) %	-	76 (8) %
Leaves	25 (14) %	37 (4) %	22 (7) %	48 (6) %

(Note: Values are presented in average, and () represent standard deviation (n=3))

4.2.7. $^{15}NO_3^-$ enrichment and isotope mixing in plants (using an enriched $^{15}NO_3^-$)

^{15}N atom percent abundances of $^{15}NO_3^-$ in recirculating water increased from natural abundance levels (Figure 4.12) to enriched levels after the additions of labeled nitrate ($K^{15}NO_3$) (Figures 4.13 and 4.14). The ^{15}N atom percent abundances of $^{15}NO_3^-$ in recirculating water remained at high levels until the end of experiments but continued to decrease toward the ^{15}N atom percent fish feed (0.3683 at%, 5.6 ‰) due to the isotope dilution of excreted nitrogen from fish feed. Nitrate was the major nitrogen sources for all the plant species (lettuce, pak choi, tomato, and chive) due to the enrichment in ^{15}N in the plants. All the plant extracts (nitrate) and tissue (organic nitrogen) enriched in ^{15}N (Figures 4.13a, 4.13b, 4.14a, and 4.14b). The differences between the enrichment patterns of ^{15}N in nitrate and organic nitrogen in each plant indicated that nitrate uptake and nitrate assimilation rates were not identical (Figures 4.13a, 4.13b, 4.14a, and 4.14b). The ^{15}N enrichment rates of nitrate were higher than those of organic nitrogen in all plants (Figures 4.13a, 4.13b, 4.14a, and 4.14b), suggesting that the plants accumulated ^{15}N of nitrate in the recirculating water into their cells and then assimilated the accumulated nitrate into organic nitrogen (nitrate concentrations = 609 (n=10), 810 (n=10), 1086 (n=12), and 893 (n=8) for lettuce, pak choi, tomato, and chive, respectively). For example, lettuce and pak choi translocated nitrate from the water and enriched the $^{15}NO_3^-$ into their cells to reach the level of $^{15}NO_3^-$ source (recirculating water) within nearly 10 days (Figures 4.13a and 4.13b). However, the lettuce and pak choi took about 17 days to transform nitrate in the recirculating water into their organic nitrogen (Figures 4.13a and 4.13b), suggesting that time required for nitrate assimilation to organic nitrogen is longer than the time for nitrate uptake into plant tissues. Moreover, the enrichment times in nitrate (10 days) organic nitrogen (17 days) in lettuce and pak choi (Figures 4.13a and 4.13b) suggested that natural abundance nitrogen

isotopic composition that previously existed in the lettuce and pak choi biomasses mixed with new nitrogen source from the enriched $^{15}NO_3^-$ source. This can be explained by nitrogen isotope mass balance, a combination of two nitrogen sources.

$$F_1 \times [N\ mass_1] + F_2 \times [N\ mass_2] = F_{mixed} \times [mass_1 + mass_2] \qquad (4.1)$$

Where N $mass_1$ is the mass of nitrogen that is taken up into a plant biomass, F_1 is the atom percent of taken up nitrogen from the water of aquaponic systems, N $mass_2$ is the mass of nitrogen at the beginning (day =0), F_2 is the atom percent nitrogen of nitrogen in a plant at the beginning (natural abundance level), and F_{mixed} is the atom percent nitrogen in a plant at the end.

Two extremes can be used to explain the isotope mixing. Firstly, consider at the day 0, plants did not take up nitrogen from the aquaponic water. Then N $mass_1$ equals 0. Thus, F_{mixed} equaled F_2, which is the natural abundance nitrogen isotope value (not enriched with the ^{15}N tracer) (see Figures 4.13a and 4.13b). Secondly, consider at the day 17, plant assimilated significant mass of nitrate in the water and amount of ^{15}N from the water. At this point, F_1 and [N $mass_1$] were extremely higher than F_2 and [N$mass_2$] (F_1 >>> F_2, and [N $mass_1$] >>> [N $mass_2$]). Then, the terms "F_2 x [N $mass_2$]" and "N $mass_2$" could be negligible from the Eq. 4.1. Thus, F_{mixed} equaled F_1 (Figures 4.13a and 4.13b). These two extremes (day 0 and day 17) show the reasons why ^{15}N atom percent in plant organic nitrogen increased over 17 days of operation. The isotope mixing for nitrate in the plant cells with a higher mixing rate can be explained by the same ways (day 0 to day 10). In conclusion, due to isotope mixing and their turnover rates, the plants required a times to elevate an enriched level of ^{15}N from a natural abundance level.

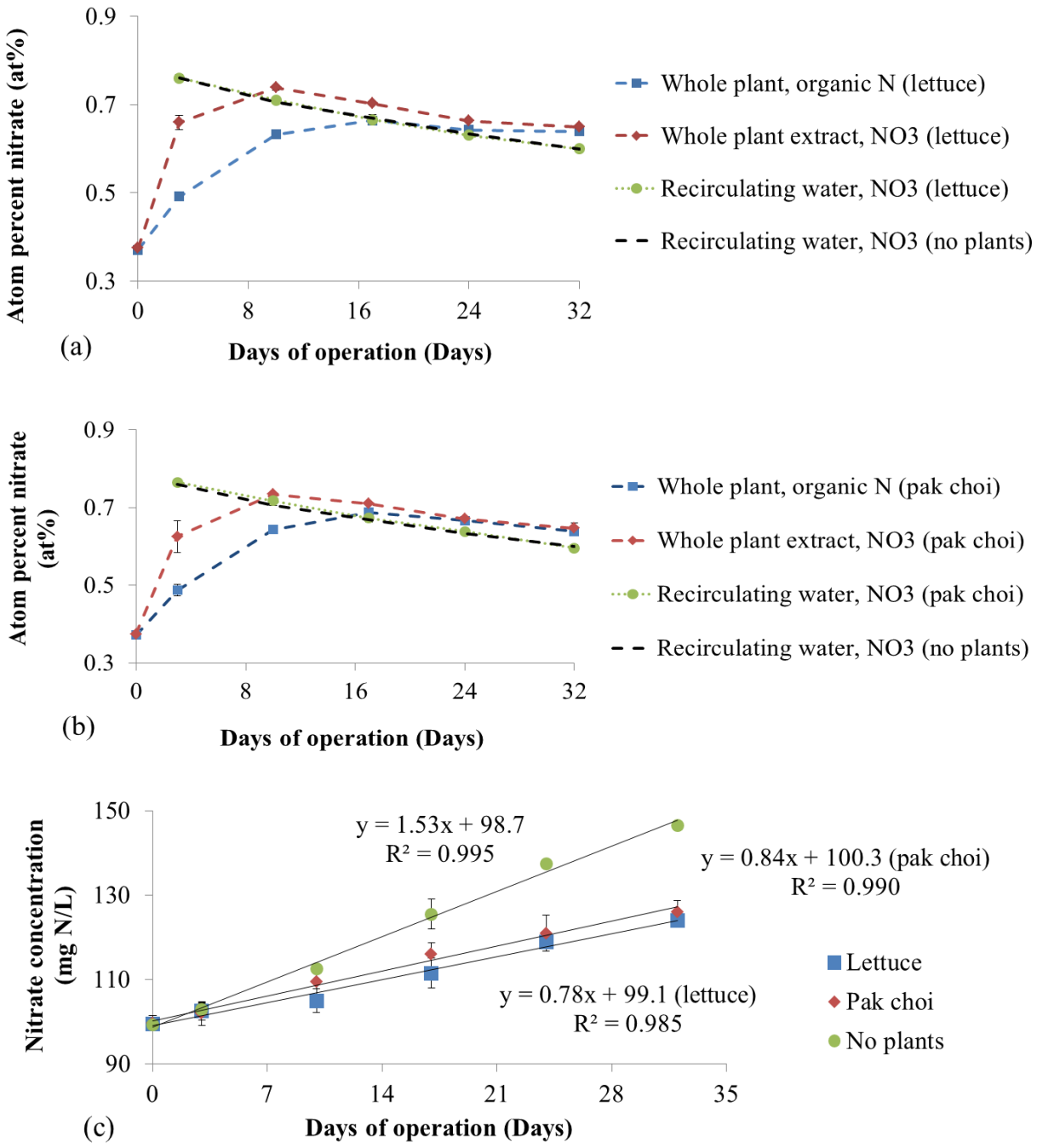

Figure 4.13. Isotope values of nitrate (atom percent), in comparison with a control (no plants), in lettuce-based (a) and pak choi-based (b) aquaponic systems. Nitrate accumulations in aquaponic systems (c)

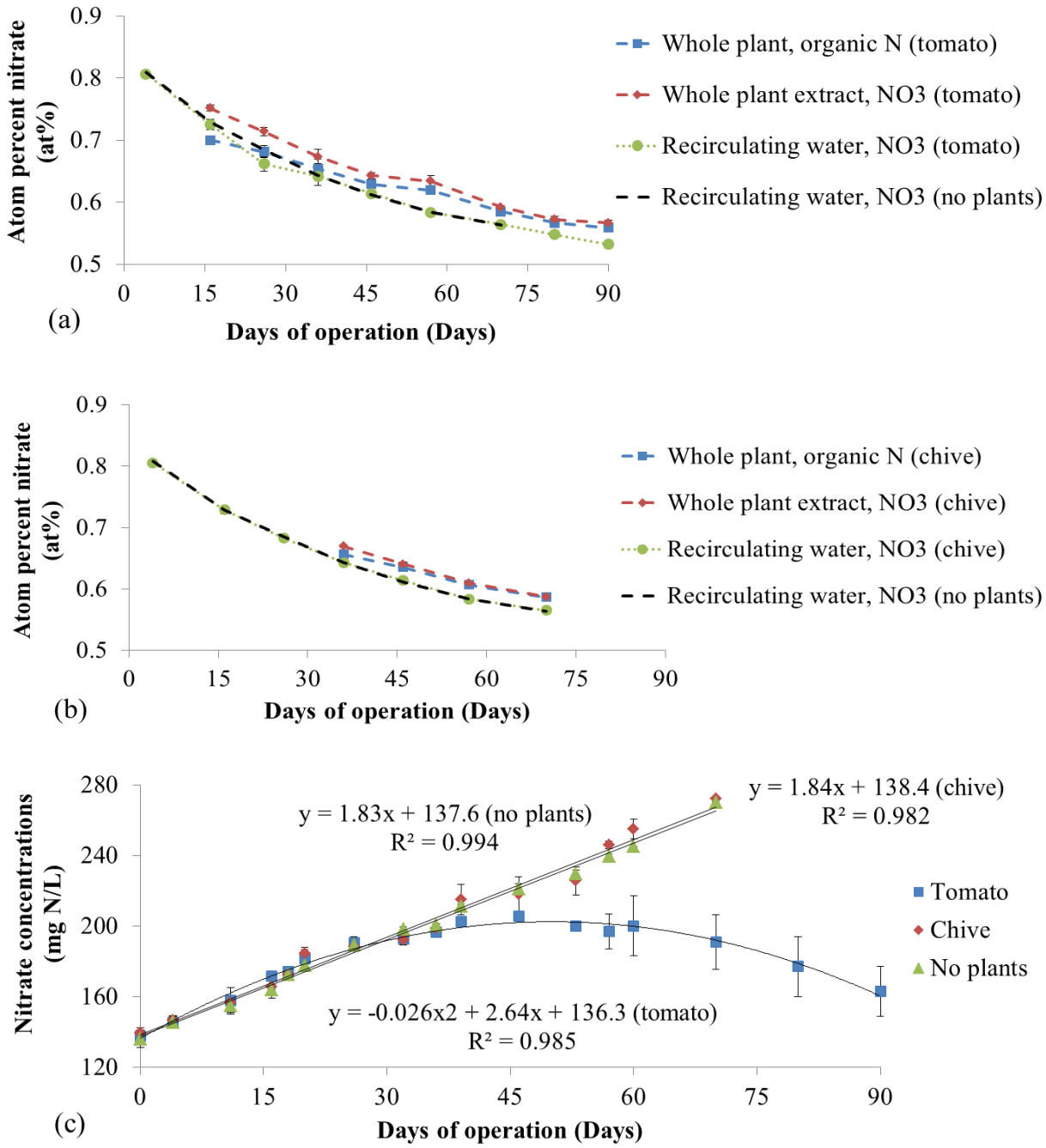

Figure 4.14. Isotope values of nitrate (atom percent), in comparison with a control (no plants), in tomato-based (a) and chive-based (b) aquaponic systems. Nitrate accumulations in aquaponic systems (c)

^{15}N enrichment in plants became significant, and natural abundance ^{15}N were not significant after day 17 in lettuce and pak choi (Figures 4.13a and 4.13b) although the isotope mixing between natural abundance ^{15}N and enriched ^{15}N remained continuing until the end of harvesting. At this point, ^{15}N from nitrate in recirculating water overwhelmed the natural abundance levels of ^{15}N in lettuce and pak choi. Nitrate and organic nitrogen in plant biomass were dependent from ^{15}N enriched nitrate in recirculating water (Figures 4.13a and 4.13b) because the plants took up nitrate from the water. The atom percent of nitrate continued to decrease with operating time. The decreases in ^{15}N in plant organic nitrogen and plant nitrate were due to the decrease in ^{15}N of nitrate in the recirculating water, where fish feed (atom percent = 0.3683 %) continuously diluted the atom percent of nitrate in the water (atom percent > 0.7300 % at day 3, Figures 4.13a and 4.13b). This depletion of nitrate atom percent in the water resulted in the depletion of nitrogen atom percent in plant extract nitrate and plant tissue organic nitrogen. If the plants stop taking up the nitrate in the water, or nitrate is no longer the nitrogen source, atom percent of nitrogen in plant tissues (organic nitrogen) and plant extracts (nitrate) will be independent from the recirculating water, not decreasing with time. Figures 4.13a, 4.13b, 4.14a, and 4.14b show that atom percent of organic nitrogen and nitrate in lettuce, pak choi, tomato, and chive were dependent from atom percent of nitrate recirculating water. Therefore, nitrate were the major source of nitrate accumulation in plants and organic nitrogen in plant tissues.

Figures 4.13c and 4.14c show nitrate concentrations, supporting that nitrate was the source of nitrogen from the recirculating water. Nitrate accumulation rates in lettuce-, pak choi-, and tomato-based aquaponic systems were lower than nitrate accumulation rates in aquaponics with no plants (controls). Nitrate accumulation rate in chive-based aquaponics did not show a significant different from aquaponic with no plants due to the slow growth and nitrogen uptake rates of chive. However, the enrichment of ^{15}N in chive extract and organic nitrogen show that chive translocated and assimilated nitrate from the recirculating water.

Lettuce, pak choi, tomato, and chive did not affect the abundance of ^{15}N in the recirculating water (nitrate source) (Figures 4.13a, 4.13b, 4.14a, and 4.14b) because the atom percent values of nitrate in recirculating water of aquaponic with plants were identical to the controls (no plants). The results confirmed that isotope effect was negligible in this enriched ^{15}N

study, and the enrichment of ^{15}N above 0.7300 at% (or 1000 ‰) was high enough to overwhelm the isotope effects that could take place in the aquaponic systems.

Although lettuce, pak choi, tomato, and chive used nitrate from the recirculating water, the atom percent of ^{15}N of the source (nitrate in recirculating water) and the products (nitrate plant extracts and organic nitrogen in plant tissues) did not contain the same isotopic value after the enriched ^{15}N overwhelming natural abundance ^{15}N. At this high ^{15}N level in which an isotope effect is negligible, it is not possible that the atom percent of the products was higher than the source. However, the reason was due to the turnover rate ($NO_3^- \rightarrow$ organic N) and isotope mixing between the enriched ^{15}N that was previously accumulated in the plant tissues and new enriched ^{15}N that came from the recirculating water, which decreased over time due to isotope mixing from fish feed (0.3683 at%) that was continuously added every day. The isotope mixing can be explained by the Eq.4.1, suggesting that isotope values in products with enriched ^{15}N were mixed by the lower ^{15}N atom percent from the recirculating water. In conclusion, all the plants in aquaponic systems recovered nitrogen from aquaculture wastewater into their biomass.

4.2.8. Nitrate uptakes and assimilation by plants and sediments (using an enriched $^{15}NO_3^-$)

Nitrate was the dominant source of nitrogen for lettuce, pak choi, tomato, and chive, but nitrate was not the significant source of nitrogen in the sediment in biofilter. Ammonia and nitrite were not the main nitrogen source for the plants because the atom percent of organic nitrogen in plant tissues were not significantly different from nitrate in plant extract at the end of each operation (Tables 4.11 and 4.12). The following equations (Eqs. 4.2 and 4.3) explain the percent ammonia and nitrate nitrogen that cooperate into plant biomass. First, assuming there was very low nitrite concentration so that nitrite uptake by plant can be negligible. Secondly, assuming ^{15}N absolutely overwhelms natural abundance nitrogen isotope level. Thirdly, isotope effect due to root efflux was negligible since all isotope effects is overwhelmed at this ^{15}N atom percent level. Then recall isotope mass balance. If plants assimilated ammonia, which was not labeled with the enriched ^{15}N from the water, atom percent of nitrate in plant extract and atom percent of organic nitrogen in whole plant will not be identical.

If both ammonia and nitrate nitrogen incorporated into plant organic N:

$$[NO_3^- \rightarrow \text{Organic N}] + [NH_4^+ \rightarrow \text{Organic N}]$$

$$F_{\text{organic N}} = (F_{NH4} \text{ x N mass}_{NH4} + F_{NO3} \text{ x N mass}_{NO3})/(\text{N mass}_{NH4} + \text{N mass}_{NO3}) \quad (4.2)$$

If only nitrate nitrogen incorporated into plant organic N:

$$[NH_4^+ \rightarrow \text{Organic N}]$$

$$F_{\text{organic N}} = (F_{NO3} \text{ x N mass}_{NO3})/(\text{N mass}_{NO3}) \quad (4.3)$$

$$F_{\text{organic N}} = F_{NO3}$$

Where N mass_{NH4} and N mass_{NO3} are the mass of ammonia and nitrate nitrogen that are taken up from the water of aquaponic systems into a plant biomass, respectively; F_{NH4} and F_{NO3} are the atom percent of taken up ammonia and nitrate in a plant, respectively; $F_{\text{organic N}}$ is the atom percent of organic nitrogen in a plant at the same time.

Figures 4.13a, 4.13b, 4.14a, and 4.14b and Tables 4.11 and 4.12 show that atom percent of organic nitrogen are not significantly different from atom percent of nitrate in plants ($F_{\text{organic N}} = F_{NO3}$). The results fell to the case that only nitrate nitrogen incorporated into plant organic N (Eq. 4.3). In the same way for nitrite, "$F_{\text{organic N}} = F_{NO3}$" confirms that nitrite was not the major nitrogen source for the plants in the aquaponic systems. The case "$F_{\text{organic N}} = F_{NO3}$" also confirms dissolved organic nitrogen and amino acids were not the major source of the nitrogen. The results were agreed to the Michaelis-Menten kinetics of nutrient uptake, which suggested that nutrient uptake rate is dependent from nutrient concentration (Bassirirad, 2000; Wongkiew et al., 2017a). Nitrate concentrations in the aquaponic systems (> 100 mg N/L) were extremely higher than TAN (< 1 mg N/L) and nitrite concentrations (< 0.3 mg/L) during the operations. Therefore, nitrate was the major significant nitrogen source in the aquaponic systems.

Nitrate and ammonia were not the major source of nitrogen in sediment from lettuce-, pak choi-, tomato-, chive-based, and plant-less aquaponic systems. Low enrichments of ^{15}N (0.3848 at% or 50.7 ‰), from $^{15}NO_3^-$ in the water, in the sediments (Tables 4.11 and 4.12) suggested that major source of the sediment was fish feces. Fish feed was not a part of sediments

because fish consumed 100% fish feed within 10 minutes after feeding. The following isotope mass balance equations show the percent distribution of fish feces into sediment. Assuming sediment is the combination between feces and biomass cells that assimilated nitrate from the water.

$$F_{sed} \times N\ mass_{sed} = F_{feces} \times N\ mass_{feces} + F_{NO3} \times N\ mass_{NO3} \qquad (4.4)$$

Where $N\ mass_{sed}$, $N\ mass_{feces}$, and $N\ mass_{NO3}$ are the mass of sediment, feces, and nitrate nitrogen that were assimilated from the water of aquaponic systems into the sediment, respectively. F_{sed}, $F_{feces,}$ and F_{NO3} are the atom percent of sediment, feces, and nitrate assimilated by biomass cells, respectively. Since sediment is the combination between feces and biomass cells that assimilated nitrate from the water, nitrogen in sediment can be written as Eq.4.5.

$$N\ mass_{sed} = N\ mass_{feces} + N\ mass_{NO3} \qquad (4.5)$$

Then substitute (4.5) to (4.4),

$$F_{sed} \times N\ mass_{sed} = F_{feces} \times N\ mass_{feces} + F_{NO3} \times (N\ mass_{sed} - N\ mass_{feces}) \qquad (4.6)$$

$$(N\ mass_{feces} / N\ mass_{sed}) \times 100 = \%\ N\ feces\ in\ sediments \qquad (4.7)$$

Rearrange the equation 4.7,

$$\%\ N\ feces = (F_{sed} - F_{NO3})/(F_{feces} - F_{NO3}) \times 100 \qquad (4.8)$$

Percent nitrogen from feces in the sediment can be calculated using the Eq. 4.8 with atom percent of sediment and average atom percent of nitrate (start and final) from the recirculating water (Tables 4.11 and 4.12). Thus, nitrogen from fish feces contributed to nitrogen in the sediments by 95.1 ± 2.3% (n = 6), and nitrate nitrogen from heterotrophic cells contributed to the sediment by 4.9 ± 2.3% (n = 6).

Table 4.11. Isotope values ($\delta^{15}N$ and atom percent) at the beginning and the end of operations in lettuce- and pak choi-based aquaponic systems, in comparison with a control (no plants)

Sources of samples	Start		Final	
	$\delta^{15}N$ (‰)	**AP (at%)**	**$\delta^{15}N$ (‰)**	**AP (at%)**
Lettuce-based aquaponic systems (32 days)				
Water from fish tank (nitrate)	1116.8* (26.0)	0.7672* (0.0094)	640.6 (21.6)	0.5595 (0.0079)
Whole plant (organic nitrogen)	12.4	0.3708	748.7 (22.7)	0.6388 (0.0082)
Whole plant extract (nitrate)	22.4	0.3745	776.7 (9.2)	0.6490 (0.0033)
Root (organic nitrogen)	-	-	535.2 (0.4)	0.5613 (0.0001)
Root extract (nitrate)	-	-	667.2 (7.7)	0.6092 (0.0028)
Sediment (organic nitrogen)	-	-	36.2 (5.9)	0.3975 (0.0021)
Pak choi-based aquaponic systems (32 days)				
Water from fish tank (nitrate)	1147.9* (19.9)	0.7835* (0.0072)	630.4 (0.6)	0.5959 (0.0002)
Whole plant (organic nitrogen)	16.5	0.3723	749.4 (25.2)	0.6391 (0.0091)
Whole plant extract (nitrate)	24.2	0.3751	771.3 (39.3)	0.6470 (0.0143)
Root (organic nitrogen)	-	-	633.3 (4.5)	0.5969 (0.0016)
Root extract (nitrate)	-	-	662.6 (39.3)	0.6076 (0.0115)
Sediment (organic nitrogen)	-	-	39.2 (4.0)	0.3806 (0.0015)
Aquaponics with no plants (control) (32 days)				
Water from fish tank (nitrate)	1114.2* (15.6)	0.7713* (0.0056)	641.1 (19.2)	0.5997 (0.0070)
Sediment (organic nitrogen)	-	-	39.1 (7.5)	0.3806 (0.0027)

Note: Values are the mean of multiple data, and values in parenthesis represent standard deviation (n = 2). * estimated from exponential regression lines of all data.

Table 4.12. Isotope values ($\delta^{15}N$ and atom percent) at the beginning and the end of operations in tomato- and chive-based aquaponic systems, in comparison with a control (no plants)

Sources of samples	Start		Final	
	$\delta^{15}N$ (‰)	AP (at%)	$\delta^{15}N$ (‰)	AP (at%)
Tomato-based aquaponic systems (90 days)				
Water from fish tank (nitrate)	1122.0 (4.2)*	0.7741 (0.0015)*	454.9 (18.9)	0.5320 (0.0069)
Whole plant (organic nitrogen)	13.1	0.3711	527.6 (4.9)	0.5585 (0.0018)
Whole plant extract (nitrate)	23.1	0.3473	549.8 (12.1)	0.5665 (0.0044)
Whole fruit (organic nitrogen)	-	-	595.2 (47.8)	0.5830 (0.0174)
Whole fruit extract (nitrate)	-	-	536.6 (3.2)	0.5617 (0.0012)
Root (organic nitrogen)	-	-	331.3 (6.7)	0.4870 (0.024)
Root extract (nitrate)	-	-	473.4 (9.3)	0.5388 (0.0034)
Sediment (organic nitrogen)	-	-	39.6 (3.9)	0.3807 (0.0014)
Chive-based aquaponic systems (70 days)				
Water from fish tank (nitrate)	1185.5 (14.8)*	0.7971 (0.0053)*	545.1 (2.5)	0.5648 (0.0009)
Whole plant (organic nitrogen)	11.2	0.3704	605.1 (0.8)	0.5866 (0.0003)
Whole plant extract (nitrate)	31.1	0.3776	606.1 (8.1)	0.5872 (0.0030)
Root (organic nitrogen)	-	-	578.4 (3.8)	0.5769 (0.0014)
Root extract (nitrate)	-	-	563.7 (4.9)	0.5716 (0.0018)
Sediment (organic nitrogen)	-	-	50.7 (2.5)	0.3848 (0.0009)
Aquaponics with no plants (control) (70 days)				
Water from fish tank (nitrate)	1193.4 (1.4)*	0.7998 (0.0005)*	541.3 (2.2)	0.5634 (0.0008)
Sediment (organic nitrogen)	-	-	50.0 (10.3)	0.3845 (0.0037)

Note: Values are the mean of multiple data, and values in parenthesis represent standard deviation (n = 2). * estimated from exponential regression lines of all data

High percent nitrogen from fish feces in the sediment also agreed with the microbial community in the biofilter (section 4.3.3). High relative abundance of *Cetobacterium* spp. were found in the biofilters and was reported in intestinal track of tilapia *Oreochromis mossambicus* (Tsuchiya et al., 2008). High amount of fish feces in the sediment of biofilter suggested that pretratment systems (preremoval) of fish feces should be installed before the attached-growth (biofilm) nitrification system to improve nitrogen use efficiency (by reducing anoxic condition) and maintain good water quality (by improving nitrification) for fish, nitrifiers, and plants in aquaponic systems. Sedmentation tank, media filtration, and multi-stage biofilter were recommended for the pretreatment of fish feces to improve nitrification efficiency (Nelson, 2008; Timmons et al., 2002).

4.2.9. Denitrification via nitrate reduction and nitrite reduction in different plant-based aquaponic systems (using an enrich $^{15}NO_3^-$)

Direct reduction of nitrite to nitrogen gas without nitrite oxidation ($NH_4^+ \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) was found as a significant pathway of nitrogen loss besides nitrogen loss via a complete denitrification from nitrate ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). The nitrogen cycle (Figures 2.3, 2.4, and 2.5) show that nitrite after ammonia oxidation at aerobic condition can be reduced by denitrifying bacteria to nitrogen gas at anoxic condition. In the aquaponic systems, the isotope mass balance of the enriched $^{15}NO_3^-$ was constructed (Eq. B.5) and nitrogen loss from the enriched $^{15}NO_3^-$ was calculated (Table 4.13). It was found that 33.7-53.4% of total nitrogen loss did not emitted from a complete denitrification ($NO_3^- \rightarrow N_2$), but emitted directly via the reduction of nitrite after ammonia oxidation (partial nitrification). This means a part of nitrite products after ammonia oxidation transformed to nitrogen gas directly under an anoxic condition at micro-anoxic environments while nitrite oxidation occurred simultaneously at aerobic environments in aquaponic systems.

Table 4.13. Percent nitrogen loss (via denitrification) from direct nitrite reduction and nitrate reduction in lettuce-, pak choi-, tomato-, chive- based aquaponics, and aquaponics with no plants

Run no.	Types of plant in aquaponics	Mass of N loss from $^{15}NO_3^-$ (gN)	Mass of N loss from feed (gN)	N loss from NO_3^- reduction (%)	Direct N loss from NO_2^- reduction (%)
1	Lettuce	12.9 (0.7)	22.7 (1.0)	57.1 (5.5)[a]	42.9 (5.5)[a]
	Pak choi	10.5 (2.2)	19.9 (1.6)	52.3 (6.5)[a]	47.7 (6.5)[a]
	No plants	13.3 (0.1)	20.2 (1.5)	66.3 (5.2)[a]	33.7 (5.2)[a]
2	Tomato	23.8 (1.8)	51.5 (8.6)	46.6 (4.3)[a]	53.4 (0.2)[a]
	Chive	23.3 (4.7)	40.3 (6.6)	61.4 (1.7)[b]	38.6 (3.6)[b]
	No plants	25.1 (5.2)	37.8 (8.8)	62.4 (0.8)[b]	37.6 (1.6)[b]

Note: Values are the mean of multiple data, and values in parenthesis represent standard deviation (n = 2). Values in Table 4.13 were calculated using the isotope information in Tables 4.11 and 4.12, and nitrogen isotope mass balance in Appendix B (Eq.B.3), and nitrogen mass balance in Appendix B (Eqs B.1 to B.4). Nitrate in plants was included in this part.

Plants had a significant effect on the direct nitrogen loss from nitrite reduction. The nitrogen loss via the $NH_4^+ \rightarrow NO_2^- \rightarrow N_2$ pathway in pak choi-based aquaponic systems was higher than aquaponics with no plants (Table 4.13, Run no. 1). The nitrogen loss via direct nitrite reduction in tomato-based aquaponic systems was higher than chive-based aquaponic systems and aquaponics with no plants (Table 4.13, Run no. 2). In contrast, nitrogen loss via a complete denitrification from nitrate was highest in aquaponics with no plants and lowest in tomato-based aquaponic systems. The results suggested that high nitrate uptake rate or high root surface area in fast-growing plants could be the factors affecting the pathways of nitrogen loss.

Nitrate uptake rate could affect nitrogen loss via nitrate reduction (a complete denitrification). Tomato took up the highest mass of nitrate from recirculating water (Figure 4.14), resulting in nitrate depletion in aquaponic recirculating water. This over uptake of nitrate by tomato decreased the nitrate concentrations and the availability of nitrate to be denitrified from aquaponic systems. As the results, the nitrogen loss from nitrate in the water of tomato-based aquaponic systems was lower than other aquaponic systems where nitrate accumulation occurred. In the contrary, a higher nitrogen loss via a complete denitrification was found at a higher nitrate accumulation rate such as in chive-based aquaponic systems and aquaponics with no plants (Figure 4.13).

Micro-anoxic zone on plant root surface area could enhance nitrogen loss after ammonia oxidation via nitrite reduction without nitrite oxidation. As the evident in nitrate accumulation and nitrogen loss (discussed specifically in sections 4.1.1 and 4.1.2), denitrification occurred in the aquaponic systems even at high DO levels. This means that micro-anoxic environments and denitrification of nitrite could occur in plant rhizosphere due to the abundance of oxygen utilizing microbes and denitrifiers (e.g., *Rhodobacterales*, *Rhizobiales*, *Sphingomonadales, and Cetobacterium*) (sections 4.3.2 and 4.3.3). A higher root surface area of plants could result in a larger zone of micro-anoxic environments due to a higher oxygen utilization rate. Once ammonia was instantaneously oxidized to nitrite around the rhizosphere by ammonia oxidizing bacteria, denitrifiers at micro-anoxic zone nearby reduced the nitrite to N_2 and N_2O directly. The results agreed with this assumption. Tomato has the highest root surface area and chive has the lowest root surface area among the four plant species (Table 4.6). Higher bacteria on root surface area of tomato enhanced the depletion of DO concentration around the rhizosphere, leading to higher nitrogen loss via the direct reduction of nitrite after ammonia oxidation up to 53.4%. Nitrogen loss via this pathway in chive-based aquaponic systems was as low as aquaponic with no plants, accounting for 33.7 to 37.6%. Supporting these statements, nitrogen loss via the direct nitrite oxidation in aquaponics with no plants at a low DO level (55.3%) was higher than a high DO level (34.7%) (Table 4.15).

The results from this part suggested that only using plants that have high root surface area to recover nitrogen from aquaponic systems might not be the best way to reduce nitrogen loss because nitrogen loss can switch between the two pathways. Maintaining high DO concentration, good water quality, and balancing nitrogen input-outputs all together are recommended to improve NUE and gain the best benefits from aquaponic systems.

4.2.10. Rapid ammonia oxidation and nitrate generation in aquaponic systems (enriched $^{15}NH_4^+$)

A rapid ammonia oxidation occurred in aquaponic systems at low DO and high DO conditions. The aquaponic systems were operated with no plants to remove the interference of plants on nitrogen transformations under two DO levels. In this experiment, $^{15}NH_4^+$ was added to the aquaponic systems instead of $^{15}NO_3^-$. Initial atom percent of nitrate (day = 0) in the recirculating water in the two systems was 0.3723 ± 0.0006 at% ($\delta^{15}N$ of NO_3^- = 16.4 ± 1.6 ‰)

(Table 4.14). However, two days following addition of $^{15}NH_4^+$ to the aquaponic systems, atom percent of nitrate in the recirculating water immediately rose to 1.2624 ± 0.0458 at% ($\delta^{15}N$ of NO_3^- = 2478 ± 128 ‰) and 1.1735 ± 0.0097 at% ($\delta^{15}N$ of NO_3^- = 2230 ± 27 ‰) in the aquaponic systems at low and high DO, respectively (Figure 4.15), with an average ammonia oxidation rate of 1.80 gN/day.

The ^{15}N atom percent of nitrate in the recirculating water decreased due to the isotope dilution of nitrate from fish feed (Figure 4.15). The atom percent nitrate at two DO conditions reached toward the atom percent fish feed (0.3683 at%) as a result from isotope mixing. Exponential regressions well fitted to the decreases in the atom percent of nitrate (Figure 4.15). According to these regressions, it could be implied that the atom percent of $^{15}NO_3^-$ at the beginning of operation, after the oxidation of $^{15}NH_4^+$, was 1.3325 and 1.2202 at% at low DO and high DO, respectively. These high values of $^{15}NO_3^-$ were due to the oxidation of $^{15}NH_4^+$ that was added at the beginning of the experiment. These values were higher than the natural abundance isotope level of nitrate before the $^{15}NH_4^+$ addition (0.3723 ± 0.0006 at%) (Table 4.14). Therefore, the results suggested that the oxidation of $^{15}NH_4^+$ to $^{15}NO_3^-$ immediately occurred once ammonia was added to the systems at low DO and high conditions.

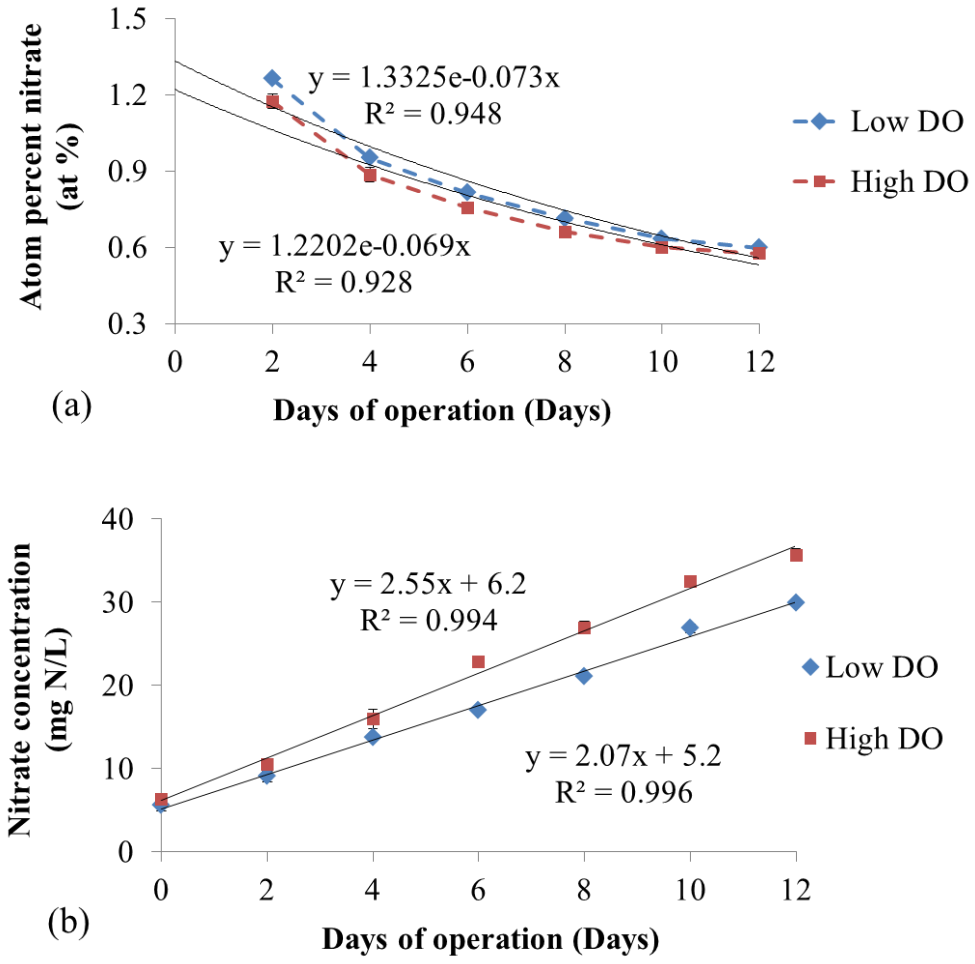

Figure 4.15. Isotope values of nitrate (atom percent) (a) and nitrate accumulation (b) in aquaponics with no plants operating low and high DO conditions

Table 4.14. Isotope values ($\delta^{15}N$ and atom percent) at the beginning and the end of operations in aquaponics with no plants at low and high DO levels

Sources of samples	Day = 0 day		Day = 12 days	
	$\delta^{15}N$ (‰)	**AP (%)**	**$\delta^{15}N$ (‰)**	**AP (%)**
Low DO levels				
Water from fish tank (nitrate)	16.4 (1.6)	0.3723 (0.0006)	634.3 (0.7)	0.5973 (0.0003)
Sediment (organic nitrogen)	-	-	113.5 (20.0)	0.4077 (0.0073)
High DO levels				
Water from fish tank (nitrate)	16.4 (1.6)	0.3723 (0.0006)	573.3 (35.1)	0.5751 (0.0128)
Sediment (organic nitrogen)	-	-	105.1 (12.4)	0.4046 (0.0045)

Note: Values are the mean of multiple data, and values in parenthesis represent standard deviation (n = 2).

4.2.11. Effects of DO on denitrification pathways (enriched $^{15}NH_4^+$)

Low DO enhanced denitrification and affected the pathways of denitrification. Nitrogen loss in aquaponic systems at low DO level was higher than high DO level (Table 4.14). Although $^{15}NH_4^+$ was the enriched ^{15}N in the systems, the atom percent of $^{15}NO_3^-$ increased rapidly within two days in aquaponics at the two DO levels. Nitrate generation rates at low DO and high DO conditions were found to be 1.34 and 1.66 gN/day, respectively. The nitrogen loss via direct nitrite reduction at low DO level was higher than the high DO level (Table 4.15), suggesting that DO also enhanced the reduction of nitrite to nitrogen gas and reduced nitrate accumulation. Results from this section suggested that avoiding anoxic zone and maintaining good water quality in aquaponic systems could reduce nitrogen loss from a complete denitrification and direct nitrite reduction.

Table 4.15. Percent nitrogen loss (via denitrification) from direct nitrite reduction and nitrate reduction in aquaponics with no plants at low DO and high DO levels

DO levels (No plants)	DO concentration (mg/L)	Mass of N loss from $^{15}NO_3^-$ (gN)	Mass of N loss from feed (gN)	N loss from NO_3^- reduction (%)	Direct N loss from NO_2^- reduction (%)
Low DO	3.8 (0.5)	2.8 (1.0)	6.2 (1.5)[a]	44.7 (6.0)[a]	55.3 (6.0)[a]
High DO	6.9 (0.2)	1.7 (0.2)	2.6 (0.4)[b]	65.3 (0.8)[b]	34.7 (0.8)[b]

Note: Values are the mean of multiple data, and values in parenthesis represent standard deviation (n = 2). Values in Table 4.15 were calculated using the isotope information in Table 4.14, nitrogen isotope mass balance in Appendix B (Eq.B.3), and nitrogen mass balance in Appendix B (Eqs B.1 to B.4). Nitrate in plants was included in this part.

4.3. Examine the ecology of functionally important living microbes and assess their contributions to nitrogen transformations in aquaponic systems

4.3.1. Microbial diversity in aquaponic systems

Plant roots and the upflow biofilter of the aquaponic systems harbored a wide range of microbial communities, especially at near neutral pH levels. In this study, combined samples represent samples from the upflow biofilter (see section 3.3). Table 4.16 shows microbial diversities in the roots and the combined samples from different plant-based aquaponic systems at near neutral pH levels. Table C.1 shows the operating information and aquaponic systems performances referring to microbial samples in this section (section 4.3), and Figure C.1 shows the nitrogen recovery linking to the microbial samples in this section.

Microbial diversity in aquaponic systems from four plant species (Table 4.16) exhibited more diverse microbial communities relative to other engineered nitrogen removal systems (see Shannon's diversity indices and Chao1, Table 4.16 vs. Table 4.17), such as simultaneous nitrification and denitrification in sequencing batch biofilm reactor (J. Wang et al., 2017), nitritation-anammox system aggregates (Chu et al., 2015), but much less microbial diversities than natural systems (Table 4.17) such as healthy soil (R. Wang et al., 2017), sea water (Q. Zhang et al., 2015), and acidic soil amended with biochar (Xu et al., 2014). In addition, the microbial diversity in the aquaponic systems was close to fresh water from poly-culture aquaculture systems (Zheng et al., 2016) (Table 4.17), suggesting that microorganisms in

aquaponics could effectively perform nitrogen transformations. Thus, due to sufficient microbial diversity in aquaponic systems, aquaponic systems have a potential for nitrogen recovery to achieve high NUE for sustainable food (Hu et al., 2015; Wongkiew et al., 2017b; Zhang et al., 2015).

4.3.2. Overview of microbial community compositions in aquaponic systems

Relative abundances of over 15 known bacterial phyla in aquaponic systems exhibited distinct microbial structures between the combined samples and plant roots (Figure 4.16). Most of the phyla were affiliated to putative heterotrophs (e.g., *Proteobacteria, Bacteroidetes*, *Acidobacteria*, *Chloroflexi*, *Chlorobi*, and *Planctomycetes*) (Persson et al., 2017) that consumed the organic matter in the aquaponic systems. *Proteobacteria*, a fast-growing r-strategist, were the most dominant phylum in both root and combined samples (Figure 4.16). Canonical correspondence analyses (CCA) (Ter Braak, 1986) showed that low pH levels (Table C.1) did not affect the shifts in microbial communities in the combined samples (Figure 4.17) (*p* value = 0.00417, adjusted $r^2 = 0.333$), but the low pH levels (Table C.1) were the major contributing factor that changed the microbial communities in the root samples (Figure 4.18) (*p* value = 0.0472, adjusted $r^2 = 0.193$). (Function anova.cca() in R was used for tests of significance.)

Table 4.16. Microbial diversity indices from combined (fish tank effluent, sediment, and biofilms) and root samples from pak choi-, lettuce-, tomato-, and chive-based aquaponic systems operated at pH range 6.8 to 7.0.

Sample	Reads	Richness	Evenness	Shannon's diversity index	Simpson's index of diversity	Chao1	ACE	Good's coverage
Pak choi (combined)	21619	390	0.67	4.00	0.940	501	478	0.996
Lettuce (combined)	51928	532	0.66	4.16	0.945	611	607	0.998
Tomato (combined)	32433	478	0.71	4.42	0.968	583	573	0.997
Chive (combined)	46251	502	0.73	4.56	0.976	567	565	0.998
Pak choi (root)	59530	567	0.71	4.50	0.972	662	640	0.999
Lettuce (root)	6816	310	0.74	4.24	0.964	391	388	0.996
Tomato (root)	37889	466	0.71	4.35	0.971	559	550	0.999
Chive (root)	20361	414	0.71	4.29	0.958	501	487	0.997

Table 4.17. Microbial diversity indices from other engineered biological systems, natural systems, and aquaculture systems

Other systems	Reads	Richness	Shannon's diversity index	Simpson's index of diversity	Chao1	ACE	Good's coverage	References
Simultaneous nitrification and denitrification in sequencing batch biofilm reactor (NaCl 10 g/L)	6167	221	2.92	0.17	226	228	0.997	J. Wang et al. (2017)
Nitritation-anammox system aggregates	46519	334	2.89	N/A	390	N/A	0.9985	Chu et al. (2015)
Healthy soil	N/A	5915	7.57	N/A	7426	N/A	N/A	R. Wang et al. (2017)
Sea water (water)	8529	979	7.8	N/A	1290	N/A	N/A	Q. Zhang et al. (2015)
Acidic soil amended with biochar and rape planting	N/A	3507	9.39	0.994	7694	N/A	N/A	Xu et al. (2014)
Black carp ponds (water)	305	N/A	5.8	0.95	396	392	N/A	Zheng et al. (2016)
Yellow catfish ponds (water)	319	N/A	5.1	0.93	417	415	N/A	Zheng et al. (2016)
Black carp ponds (sediment)	981	N/A	8.3	0.99	1287	1295	N/A	Zheng et al. (2016)
Yellow catfish ponds (sediment)	1041	N/A	8.4	0.99	1387	1381	N/A	Zheng et al. (2016)

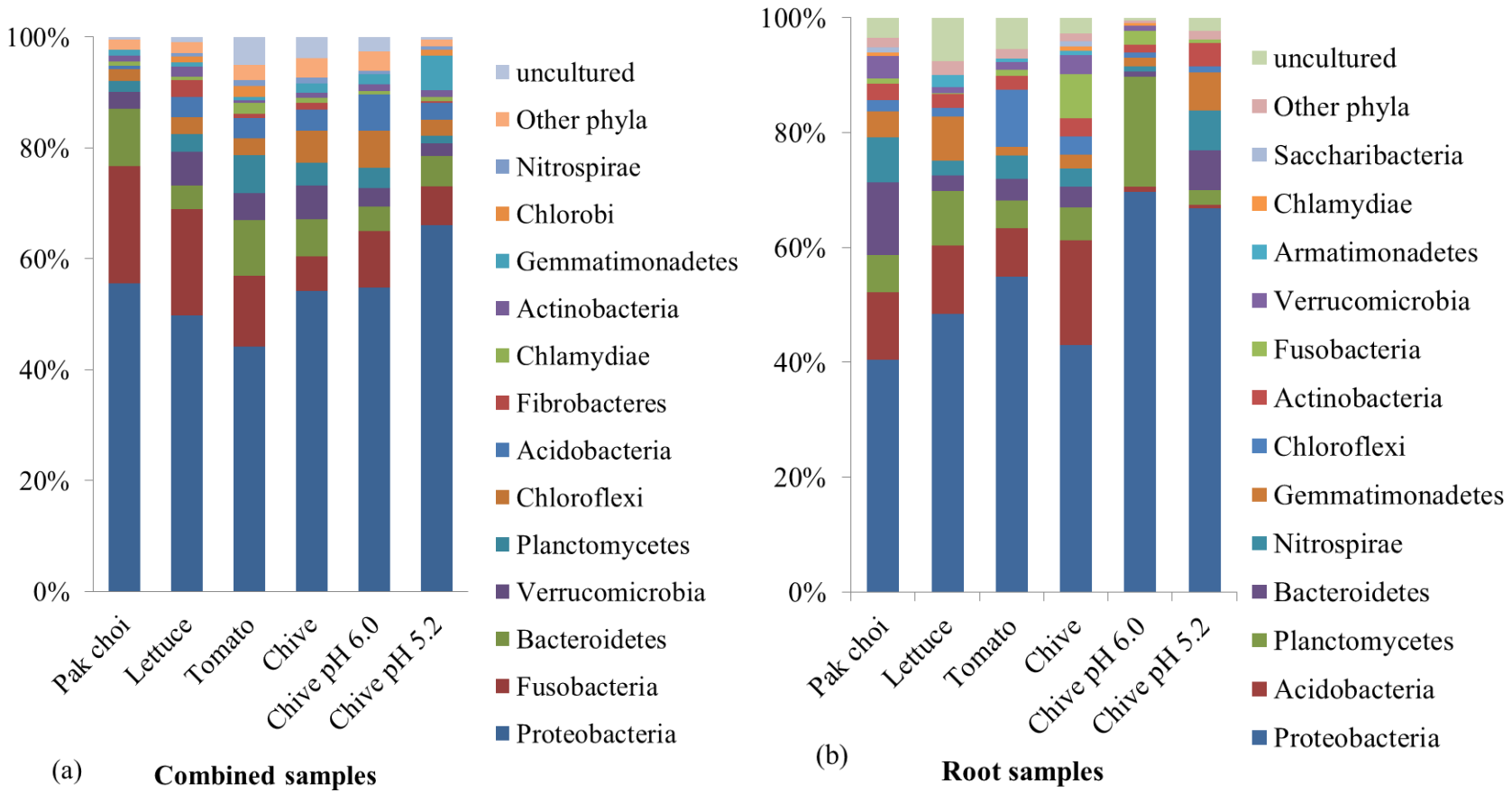

Figure 4.16. Bacterial phyla from combined samples (fish tank effluent, sediment, and biofilms) (a.) and root samples (b.) of different plant-based aquaponic systems and pH levels (Phyla with relative abundance below 0.5% were assigned as other phyla

In the combined samples, TAN concentrations positively correlated to the relative abundance of phylum *Gemmatimonadetes* (Figure 4.16a), which contributed up to 6.2% of relative abundance at pH of 5.2, from 0.5-1.7% at near neutral pH levels (pH 6.8-7.0). Other major phyla from the combined samples (Figure 4.16a) include *Proteobacteria*, *Fusobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Planctomycetes*, and *Chloroflexi*, accounting for 44.2-64.1%, 6.2-21.2%, 4.3-10.4%, 2.3-6.1%, 1.4-6.9%, and 2.1-6.7%, respectively. *Proteobacteria* and *Nitrospirae* in the combined samples were likely independent of pH levels (Figure 4.17).

In plant roots, phyla at near neutral pH levels among four plant species (Figure 4.16b) such as *Proteobacteria*, *Acidobacteria*, *Planctomycetes*, *Bacteriodetes*, *Nitrospirae*, and *Gemmatimonadetes* accounted for 40.4-54.9%, 8.5-18.3%, 4.8-9.6%, 2.6-12.6%, 2.6-7.9%, and 1.6-7.8%, respectively. However, at low pH levels, these phyla accounted for 66.9-69.6%, 0.5-0.9%, 2.7-19.2%, 0.9-6.9%, 0.9-7.0, and 1.4-6.7%, respectively, indicating the microbial shifts between neutral and low pH levels (Figures 4.16b and 4.18). The abundances of some dominant phyla in plant roots, such as *Acidobacteria* and *Chloroflexi*, were dominated by *Proteobacteria* when pH dropped to a low level (pH of 5.2) (Figures 4.16b). Microbial structures from the plant roots were more sensitive to the low pH levels relative to those from combined samples, suggesting that plant roots in aquaponic systems could be the key operating or limiting factor to control effective nitrogen transformations and recovery.

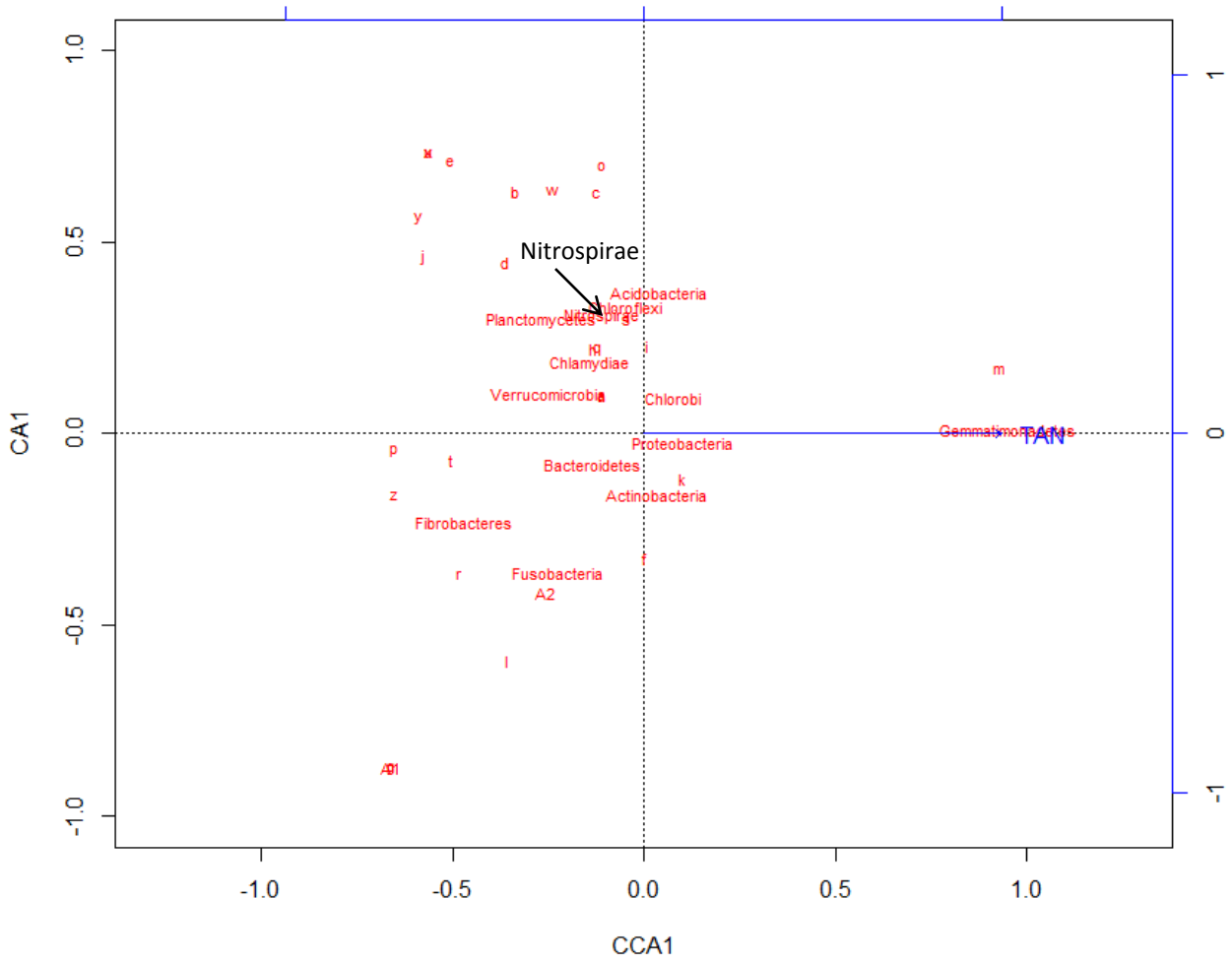

Figure 4.17. Canonical correspondence analysis plot (*p* value = 0.025, adjusted r^2 = 0.128) based on relative abundances of all microbial phyla and TAN concentration from the combined samples in aquaponic systems. Symbols (characters a to z, A1, and A2, see Table C.3 for full name) represent "other phyla in Figure 4.16a" from combined samples (fish tank effluent, sediment, and biofilm).

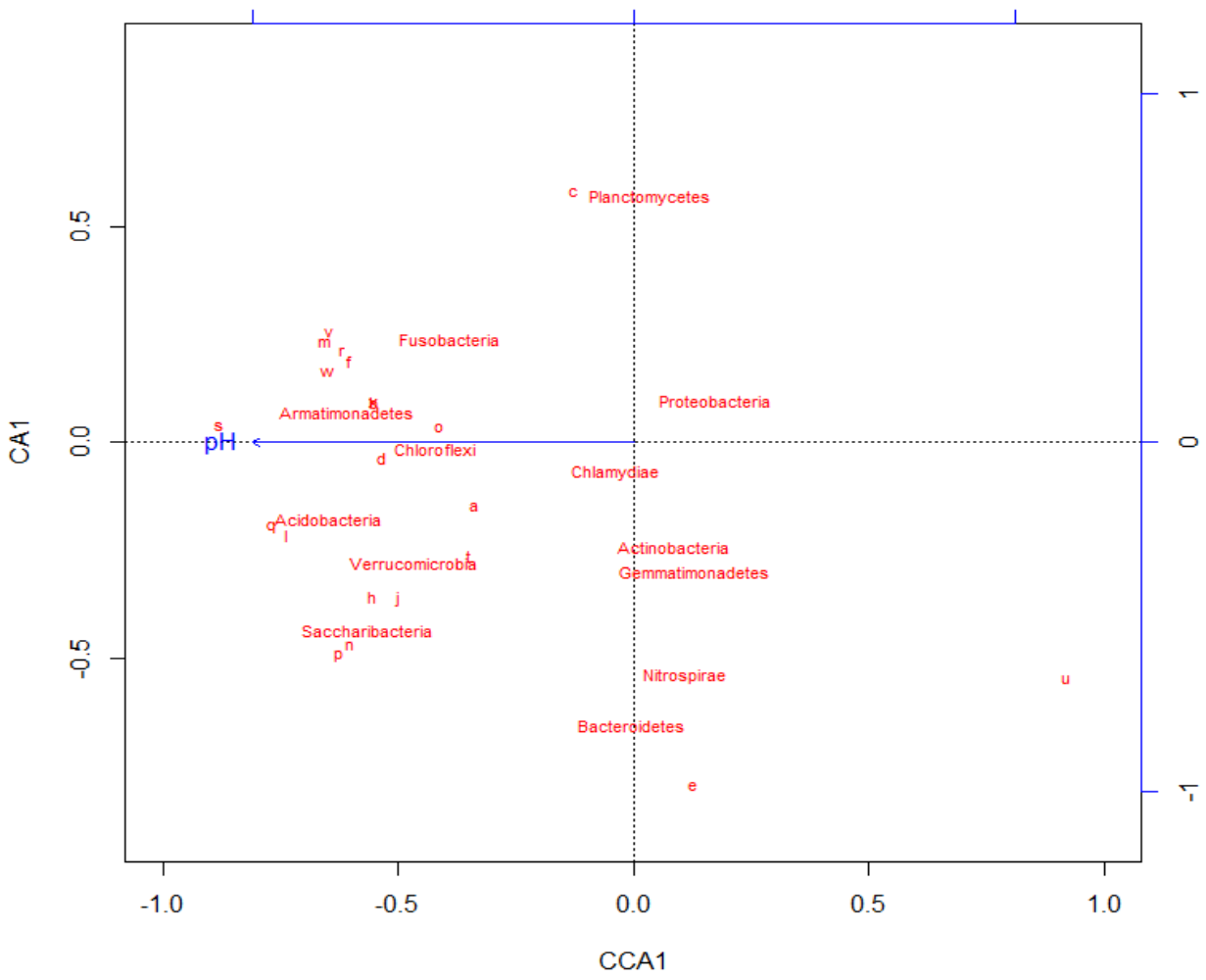

Figure 4.18. Canonical correspondence analysis plot (*p* value = 0.09306, adjusted r^2 = 0.0945) based on relative abundances of all microbial phyla and pH from root samples in aquaponic systems. Symbols (characters a to y, see Table C.3 for full name) represent "other phyla in Figure 4.16b" from root samples.

In the combined samples at near neutral pH levels, *Fusobacteria* (mostly genus *Cetobacterium*, Figure C.2b) dominated over *Acidobacteria* (Figure 4.16a). However, in the root samples at near pH levels, *Acidobacteria* dominated over *Fusobacteria* (Figure 4.16b). The abundance of *Acidobacteria* in the roots decreased at low pH levels (5.2-6.0) because *Acidobacteria* subgroup 6, which positively correlated to pH levels, were the most abundant among all *Acidobacteria* subgroups in the plant roots in the aquaponic systems (Figure C.3b) (Da Rocha et al., 2013; Jones et al., 2009; Kielak et al., 2016). *Acidobacteria* subgroup 6 were predominantly found in both nutrient-rich soils, and terrestrial and marine environments; however, their ecological roles are still unknown (Kielak et al., 2016). *Proteobacteria, Verrucomicrobia, Bacteriodetes, Actinobacteria, Planctomycetes, and Cholorofexi* (facultative biofilm forming bacteria (Persson et al., 2017)) found in the aquaponic systems were also the major shared phylum found in roots and biofilters in a pilot-scale aquaponics (Schmautz et al., 2017), activated sludge system (Ye et al., 2012), simultaneous nitrification-denitrification process (J. Wang et al., 2017), partial nitritation-anammox biofilms (Persson et al., 2017), agricultural soils (Fierer et al., 2012), and acidic soils (Xu et al., 2014), suggesting that understanding of nitrogen transformations in other systems could facilitate the improvement of NUE in aquaponic systems.

Class *Alpha-, Beta- and Gammaproteobacteria* predominated in combined and root samples (Figures C.2a and C.3a). The abundance of *Betaproteobacteria*, a member of ammonia oxidizers (Philippot et al., 2013), in root samples decreased when pH changed to low levels (Figure C3.a), but their abundance did not change in the combined samples (Figure C.2a). At the order level in combined and root samples, *Burkholderiales* (bacteria assimilating carbon substrates from root efflux (exudates) (Philippot et al., 2013)) and *Rhodocyclales* were the major members of *Betaproteobacteria* order (Figures C.4b and C.5b). *Rhodobacterales, Rhizobiales* and *Sphingomonadales* were the dominant members of *Alpha-proteobacteria* order (Figures C.4a and C.5a), which are commonly found in soil rhizosphere. These orders were reported to be the major contributors to denitrification (Bouali et al., 2013; Lu et al., 2014a; Persson et al., 2017). The presence of *Nitrosomonadales (Betaproteobacteria)* in combined and root samples (Figures C.4b and C.5b) also indicated that AOB were found ammonia-oxidizing bacteria in roots and biofilters of aquaponic systems (Schneider et al., 2015).

4.3.3. Unique roles of bacteria on nitrogen transformations in aquaponic systems

The microbial community compositions in chive, pak choi, lettuce and tomato roots were quite similar at near neutral pH (Figures 4.19 and C.6). (Figure 4.19 shows only the top 14 most abundant genera and family, and Figure C.6 shows a heatmap of all OTUs with relative abundances above 1 %.) *Nitrospira* spp. was the dominant species. *Comamonadaceae*, which was found in denitrification process of wastewater treatment and organic farming systems, indicated that nitrogen loss via denitrification is unavoidable in rhizosphere zone despite high DO in the grow beds (Lu et al., 2014b; Pershina et al., 2015). *Plesiomonas* spp. (facultatively anaerobic chemoorganotrophs in aquatic environmennts (Chopra and Galindo, 2007)), *Rhodobacteraceae* (aerobic chemoheterotrophs in aquatic environments (Pujalte et al., 2014)), and PHOS-HE51 (phosphorus removal bacteria in wastewater treatment (Ouyang et al., 2017)), were also found in both combined and root samples, suggesting that transformations of phosphorus and carbon occurred under aerobic and micro-anoxic environments throughout the aquaponic systems (Figure 4.19).

Microbial communities on chive roots at different pH levels (5.2-7.0) were not affected by DO depletions because DO concentrations in rhizosphere zone were maintained equally high (DO = 5.80 ± 0.33 mg/L, as described in section 3.2.3). Microbial community compositions (major genus and family) from the chive roots at pH 5.2-6.0 were distinct from the chive roots at near neutral pH level. Results suggested that pH was more important than plant species on affecting the microbial community shifts and the nitrogen transformations. Microbial community compositions in plant roots at low pH levels were different from the combined sample (biofilters) (Figure 4.19), but the two components facilitated nitrification, suggesting different functions of plant roots and biofilters in nitrogen transformations.

Low pH levels had a slight impact on microbial communities in the combined samples from different plant-based aquaponics. From all combined samples, *Cetobacterium* spp. (anaerobe) and *Comamonadaceae* (aerobe) were dominant. *Cetobacterium* spp., a bacterium producing acetic acid from glucose (Foster et al., 1995), is found in the intestinal tract of freshwater fish (e.g., *Oreochromis mossambicus*) (Tsuchiya et al., 2008). This genus could reduce nitrate during denitrification (Foster et al., 1995; Tsuchiya et al., 2008) and contribute to

the nitrogen loss from the sediment of biofilters during nitrogen transformations in aquaponic systems.

In the roots at the low pH levels, *Thermomonas* spp., *Mesorhizobium* spp., *Gemmata* spp., *Pseudolabrys* spp. (organic acids utilizing bacteria found in soils (Kämpfer et al., 2006)), *Asticcacaulis* spp. (obligate aerobic chemoorganotrophs found in freshwater with low concentration organic nutrients (Stolp, 1988)), *Sphingomonadaceae* (chemoorganotrophs found in soil, plant roots, and activated sludge (Kosako et al., 2000)), *Gemmatimonadaceae* (mesophilic aerobes found in of a large range of nutrient concentrations in soil (DeBruyn et al., 2011; Zhang et al., 2003), and *Xanthobacteraceae* (denitrifiers (Persson et al., 2017)) were dominant (Figure 4.19). *Thermomonas* spp. are filamentous aerobic chemoorganotrophs that do not reduce nitrate to nitrite and nitrogen, and do not usually utilize carbohydrate (Denner et al., 2015), suggesting the presence of several organic compounds in the aquaponics. *Mesorhizobium* spp. was reported to utilize ammonium, nitrate, and urea, and can penetrate plant roots and promote root nodules for nitrogen fixation in some plants (Chen et al., 2015), suggesting that nodule-forming plants (e.g., legumes) could be grown together with other plants in aquaponics to enhance NUE at these low pH levels (5.2-6.0), which increase the nutrient availability for plant uptake (Resh, 2013). Although pH range of 6.0-8.0 is optimal for *Mesorhizobium* spp. (Chen et al., 2015), higher abundance was found at low pH of around 5.2-6.0 in our aquaponic systems, suggesting a symbiotic relationship between plant roots and bacteria at low pH levels. The abundance of *Gemmata* spp., an aquatic aerobic chemoorganotroph (Chouari et al., 2003; Fuerst et al., 2015) reported in acidic environments (Kulichevskaya et al., 2017), such as bogs and wastewater treatment plants (Wang et al., 2002), indicating that high DO was available for aerobic heterotrophs and plant roots around rhizosphere in the aquaponic grow beds. Interestingly, the relative abundances of *Nitrospira* spp. and *Gemmatimonadaceae* bacteria in plant roots did not decrease at the low pH level of 5.2 from the neutral pH levels (Figure 4.19). The reason could be due to their physiological adaptation to the acidic pH conditions (Gieseke et al., 2006). The abundance of *Nitrospira* spp. suggested that plant roots in aquaponics facilitated nitrite oxidation even though the pH dropped following nitrification of ammonia generated by fish.

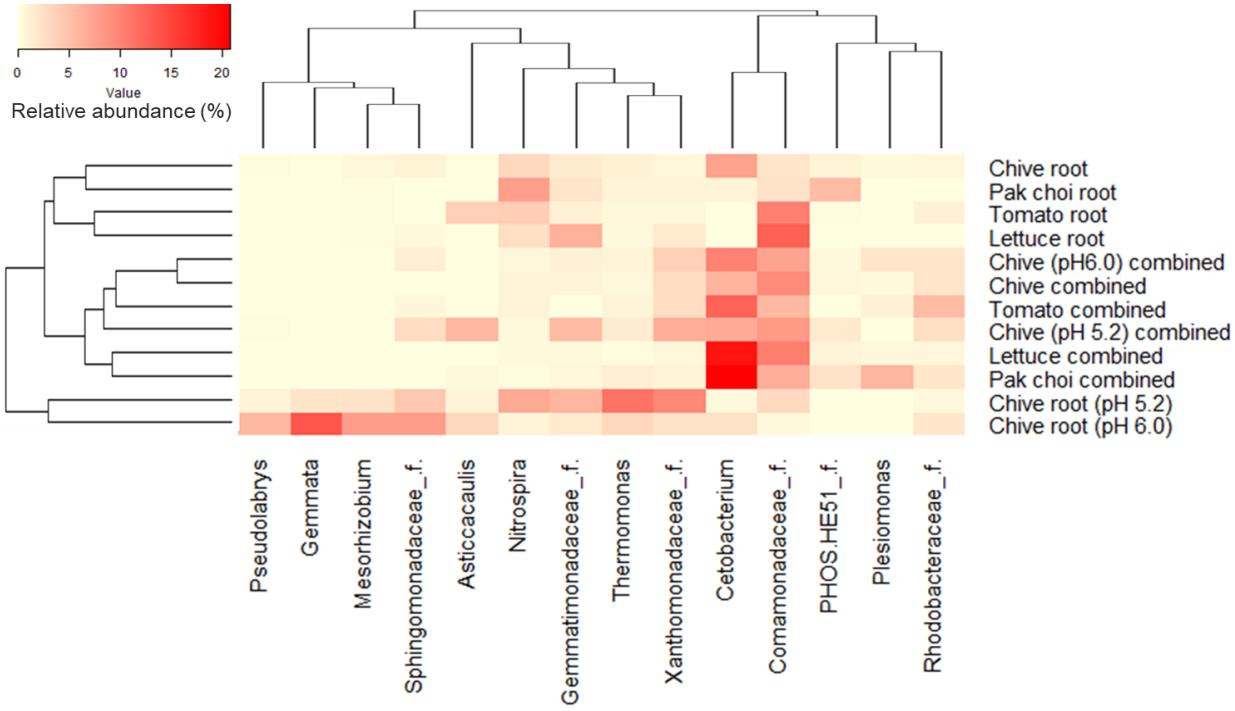

Figure 4.19. Heatmap of microbial community compositions (top 14 most abundant genera and family (.f.)) and dissimilarities (Bray-Curtis) among all samples (combined and root samples) from different plant-based aquaponic systems and pH levels

4.3.4. Abundance of nitrifying and heterotrophic bacteria in aquaponic systems

Plant roots and three subcomponents of the upflow biofilter (biofilm, fish tank effluent, and sediment) were separately studied by qPCR for further insights on specific microbial community in this section. Nitrifying bacteria in all aquaponic subcomponents and plant root were not the most predominant species, as shown by the relative abundance of nitrifiers to eubacteria (Figure 4.20). Relative abundances of AOB (*amo*A) and NOB also indicated that AOB and NOB played important roles in nitrification although they were outcompeted by other bacteria (Zou et al., 2016b). The results were in agreement with the COD concentrations (Table C.1), which facilitated facultative heterotrophic denitrification and thus contributed to the nitrogen loss in the aquaponic systems (see sections 4.3.2. and 4.3.3. for microbial community using16S rRNA gene sequencing) (Pynaert et al., 2004; Wongkiew et al., 2017b; Zou et al., 2016b). Although anammox bacteria were previously detected in other natural aquatic and engineered aquaculture environments in which nitrification and denitrification occurred

simultaneously as in the aquaponic systems (Kuenen, 2008; Pynaert et al., 2004; van Kessel et al., 2011; Vlaeminck et al., 2009; Wongkiew et al., 2017b), the abundance of anammox bacteria was not significant in the aquaponic systems due to the outgrowth of aerobic ammonia oxidizers (Figure 4.20) outcompeting for ammonia oxidation (Kuenen, 2008).

At near neutral pH levels (pH 6.8-7.0), the abundances of AOB and NOB in sediment, fish tank effluent, biofilm, and plant roots varied slightly (Figure 4.20), suggesting that those nitrifiers were likely independent of the plant species in the aquaponic systems. In contrast to other conventional biological nitrogen removal systems, *Nitrospira* spp. in the aquaponic systems were more predominant than *Nitrobacter* spp. and AOB (Figure 4.20) despite their higher growth rates (Cabrol et al., 2016; Hu et al., 2015). *Nitrospira* spp. (K-strategist) thrived and outgrew *Nitrobacter* spp. (fast-growing r-strategist) and AOB in this study due to low nitrite and ammonia concentrations, which resulted in nitrite limitation for *Nitrobacter* spp. (Blackburne et al., 2007; Nogueira and Melo, 2006). Moreover, *Nitrospira* spp. was likely more predominant in plant roots and sediment than in the fish tank effluent and biofilms in the biofilters (Figure 4.20, Table D.1). This could be attributed to perpetual accumulation of sediment over months in aquaponic biofilters that resulted in rapid oxygen depletion (Wongkiew et al., 2017b). The low DO condition in sediment resulted in an anoxic environment that suppressed *Nitrobacter* spp. more severely than *Nitrospira* spp. due to the lower oxygen and nitrite affinities of *Nitrobacter* spp. (Downing and Nerenberg, 2008; Wongkiew et al., 2017b). High abundance of *Nitrospira* spp. was also reported in the rhizosphere of water-saturated soils, wetlands, and grassland soils (DeAngelis et al., 2009); however, further studies are needed to elucidate the positive response of *Nitrospira* spp. to plant roots.

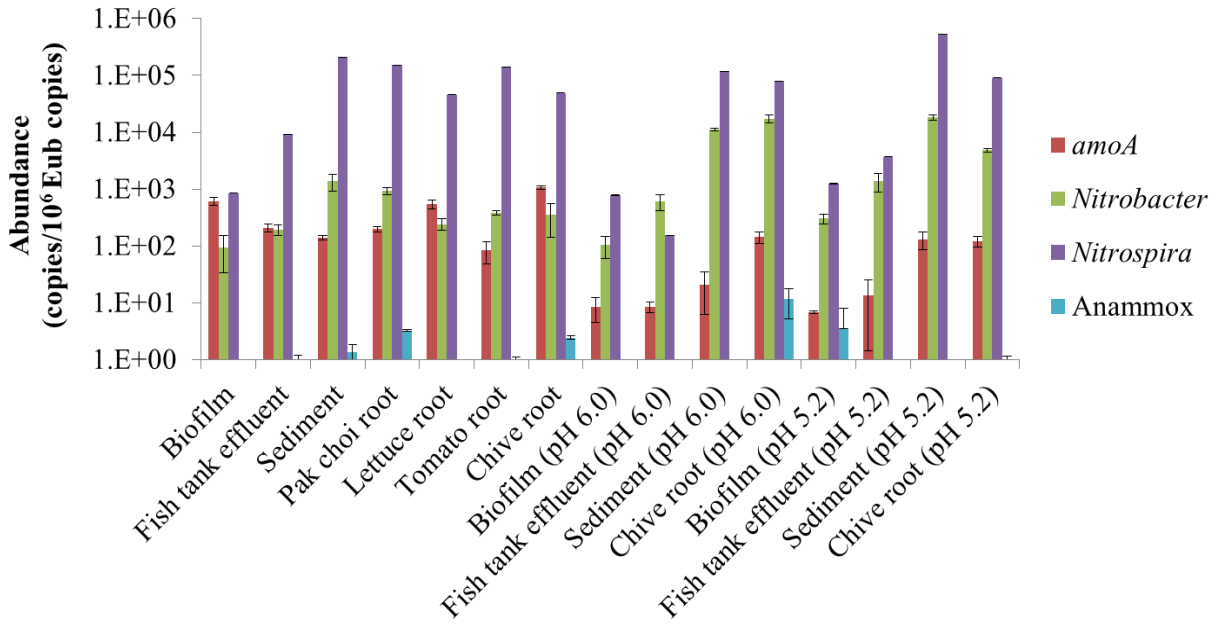

Figure 4.20. Relative abundances of nitrifying and anammox bacteria over eubacteria in different aquaponic components and pH levels. Aquaponic systems operated at near neutral pH levels (6.8-7.0) were not marked by parentheses.

Low pH levels (5.2 and 6.0) reduced the relative abundance of AOB (*amoA*) in fish tank effluent and biofilm (Figure 4.20 and Table D.2). At low pH levels, the abundance of AOB decreased by about 10-fold as compared to that in near neutral pH (Figure 4.20), increasing TAN concentrations (Table C.1). In contrast, the abundance of *Nitrobacter* spp. in sediment and chive roots at lower pH levels (5.2 and 6.0) was higher than those at neutral pH level (Tables D.3 and D.4) by about 10-fold. This range of low pH levels (5.2-6.0) resulted in a decrease in nitrite concentrations (Table C.1), suggesting that the low pH levels could positively affect only *r*-strategist nitrite oxidizers in the plant roots and sediment. The findings were in agreement with that of (Zou et al., 2016b) who reported that a decrease in pH level from 7.5 to below 6.0 significantly reduced copy numbers of genes encoding ammonia monooxygenase (AMO), nitrite reductase (NiR), and nitrous oxide reductase (N_2OR) in fish tank effluent and biofilm of clay biofilter media (perlite) in a media-based aquaponic system. Interestingly, the abundances of NOB on plant roots did not decrease at low pH levels when compared to neutral pH levels (Figure 4.20 and Tables D.6), suggesting that increasing the total root surface area could increase the surface area of NOB on plant roots for maintaining low nitrite concentration in aquaponic

systems under low pH levels of 5.2-6.0. Although the abundances of NOB in sediment were as high as in roots (Figure 4.20), accumulating the sediment in aquaponic biofilters is not recommended because this strategy will cause a rapid DO depletion and anoxic condition that enhances denitrification thereby resulting in significant nitrogen loss.

Despite differences in relative abundances from qPCR and 16S rRNA amplicon due to different sets of primers, the results of both 16S rRNA amplicon and qPCRs supported the findings on the microbial communities in the aquaponic systems (Park et al., 2017; Ye et al., 2012). While low pH levels shifted the microbial communities in roots, the abundance of *Nitrospira* spp. did not decrease.

4.4. Investigate the greenhouse gases emissions from an aquaponic system, with specific emphasis on nitrous oxide (N_2O) emission

4.4.1. Nitrous oxide emissions from aquaponic systems

N_2O emissions in aquaponic systems varied from 18.2 to 24.1 mg N/day (Table 4.18). Lettuce-, pak choi-, tomato- and chive-based aquaponic systems emitted N_2O with N_2O emission rates of 23.2, 20.4, 24.1, and 20.0 mg N/day, respectively. These values accounted for N_2O conversion ratios (N_2O emitted relative to N input) of 0.96%, 0.82%, 0.99%, and 0.80%, respectively. Plant species (pak choi, lettuce, tomato, and chive) in the floating-raft aquaponic systems did not significantly affect N_2O emissions (Table 4.18). For example, tomato-based aquaponic systems emitted the highest N_2O emission, accounting for 24.1 mg N/day or 0.99% of nitrogen input. N_2O conversion ratios from aquaponics with no plants varied from 18.2 to 19.7 mg N/day, which were equivalent to 0.75 to 0.81% of nitrogen input (Table 4.18). N_2O emission from aquaponics with no plants (run no.1) was significantly lower than the lettuce-based aquaponics systems; however, N_2O from aquaponics with no plants (run no.2) was not significant different from chive- and tomato-based aquaponic systems (Table 4.18). These results showed that plant species in aquaponic systems slightly enhanced N_2O emission rates but not potent enough to increase N_2O to a level of significant difference. Although N_2O emissions from aquaponic systems with plants (run no. 2) were not different from aquaponics with no plants, microbial community in plant roots could play dominant roles on nitrification and denitrification

(section 4.3) (Hu et al., 2015), leading to dissolved N_2O in recirculating water and subsequent N_2O emission to the atmosphere.

Table 4.18. Nitrous oxide (N_2O) emissions and N_2O conversion ratios from different plant-based aquaponic systems

Run no.	Type of Aquaponics	Fish tank (mg N/day)	Biofilter (mg N/day)	N_2O emission (mg N/day)	N_2O conversion ratio (%)
1	Pak choi-based aquaponics	15.2 (1.4)^{ab}	5.3 (0.8)^{a}	20.4 (1.8)^{a,b}	0.84
	Lettuce-based aquaponics	18.1 (1.6)^{a}	5.1 (0.6)^{a}	23.2 (2.0)^{a}	0.96
	Aquaponics without plants	15.1 (1.5)^{b}	3.1 (0.4)^{b}	18.2 (1.8)^{b}	0.75
2	Chive-based aquaponics	16.8 (2.3)^{a}	3.2 (0.5)^{a}	20.0 (2.7)^{a}	0.82
	Tomato-based aquaponics	20.7 (2.1)^{a}	3.3 (0.5)^{a}	24.1 (2.4)^{a}	0.99
	Aquaponics without plants	16.5 (2.1)^{a}	3.2 (0.5)^{a}	19.7 (2.6)^{a}	0.81

(Note: Values are the mean of multiple data (n = 4), and values in parenthesis represent standard deviation. The superscripts a and b represent statistically different ($p < 0.05$))

N_2O emission ratios in this study were lower than N_2O emission ratios from other aquaponic systems in other studies. In other floating-raft aquaponic systems, N_2O conversion ratios were reported at 1.5 and 1.9% in tomato- and pak choi-based aquaponic systems (Hu et al., 2015). Other studies showed that N_2O emission ratios of 1.3 to 1.6% emitted from media-filled (flood-and-drain) aquaponics with pak choi as growing plants, and a N_2O emission ratio of 1.3% was found in aquaculture systems (Hu et al., 2013; Zou et al., 2016a). In fact, N_2O emission ratio depended on several factors such as fish stoking density, pH, DO concentration, season, temperature, and water quality (e.g., ammonia, nitrite, and nitrate concentrations) (Fang et al., 2017; Hu et al., 2012; Zou et al., 2016b). For example, N_2O emission ratios from media-filled aquaponics accounted for 0.6%, 1.6%, and 2.0% when the aquaponics were operated pH levels of 9.0, 7.5, and 6.0, respectively (Zou et al., 2016b). Therefore, N_2O emission ratios from the floating-raft aquaponic systems in this study were lower than the other studies due to higher DO concentrations in fish tank and recirculating water, lower nitrite concentrations, and a lower constant feeding rate (35 g/day in this study). In conclusion, plants in aquaponic systems did not

have a potent effect on reducing N_2O emissions as hypothesized in section 3.2.4 and previously reported (Hu et al., 2012; Wongkiew et al., 2017a).

Plant species did not affect N_2O emission rates in both fish tanks and downflow biofilters (biofilter 2) (Table 4.18, run no.1 and 2). Since the four plant-based aquaponics and aquaponics with no plants were operated at the same operating condition, it can be implied that plant species did not reduce N_2O emissions due to the same levels of water quality in aquaponics, such as TAN, nitrite, and COD concentrations. Table 4.19 shows that water quality parameters, except nitrate concentration, in the four plant-based aquaponic systems and aquaponics with no plants operated were not significantly different. Thus, N_2O emissions could be more affected by water quality (e.g., DO, COD, and nitrite concentrations) rather than only using plant species to assimilate nitrogen (nitrate) from aquaponic systems (Hu et al., 2012).

Fish tanks (aerated surface) generated higher N_2O emissions compared to the downflow biofilter (non-aerated surface) (Table 4.18). Water in fish tanks was aerated with aeration rate of 10 L/minutes, while water in the downflow biofilter dropped from lateral pipes to throughout plastic media in the biofilters (12 inches) with a flow rate of 1.55 L/minute (HLR = 1.5 m^3/m^2-day) (Figure 4.21). Other study showed a similar result that N_2O emission rate from an aquaponics with continuous aeration was higher than that of aquaponics with intermittent aeration (Fang et al., 2017). Results implied that turbulence enhanced N_2O stripping from water; however, aerodynamic analyses or mass transfer of N_2O gas stripping from the fish tank and the downflow biofilter should be modeled to elucidate this evidence. Dissolved N_2O was generated at anoxic condition and could be mainly due to denitrification process. (Sections 4.1.2 and 4.2.9 supported that denitrification was a major pathway of nitrogen loss.) Thus, oxygen transfers in fish tank (aerating surface) and downflow biofilter (non-aerating surface) were not a cause of dissolved N_2O generation, but mixing for improving oxygen transfer in these aerating systems enhanced N_2O emission via turbulence. Optimal aeration rate should be considered when engineers need to reduce N_2O emission but maintaining high DO concentration in aquaponic systems.

Table 4.19. Water quality parameters (nitrogen and COD concentrations, and NO_3^- accumulation rates) in pak choi-, lettuce-, tomato- and chive-based aquaponic systems, and aquaponics with no plants

Run no.	Plant types	TKN (mgN/L)	TAN (mgN/L)	NO_2^- (mgN/L)	NO_3^- accumulation rate (mgN/L/d)*	COD (mg/L)
1	Pak choi	8.7 (0.9)	1.0 (0.2)	0.21 (0.05)	1.21 (0.04)	66.4 (3.1)
	Lettuce	8.1 (1.2)	0.9 (0.2)	0.20 (0.06)	1.27 (0.04)	63.9 (4.1)
	No plants	8.6 (1.1)	0.9 (0.2)	0.21 (0.05)	1.81 (0.02)	61.5 (2.9)
2	Tomato	8.3 (1.1)	1.1 (0.2)	0.27 (0.09)	$-1.14(10^{-2})t^2 + 1.89t + 65.7$	70.3 (8.8)
	Chive	8.2 (1.2)	1.0 (0.1)	0.24 (0.07)	1.66 (0.03)	69.7 (6.8)
	No plants	7.7 (1.1)	1.2 (0.2)	0.24 (0.07)	1.72 (0.01)	69.5 (5.8)

(Note: Values are the mean of multiple data for TKN, TAN, NO_2^- and COD concentrations (n = 15 for pak choi and lettuce, n= 21 for tomato and chive), NO_3^- accumulation rate (n = 3), DO concentrations (n = 35 for pak choi and lettuce, n= 90 for tomato and chive). Values in parenthesis represent standard deviation. * NO_3^- accumulation rate.)

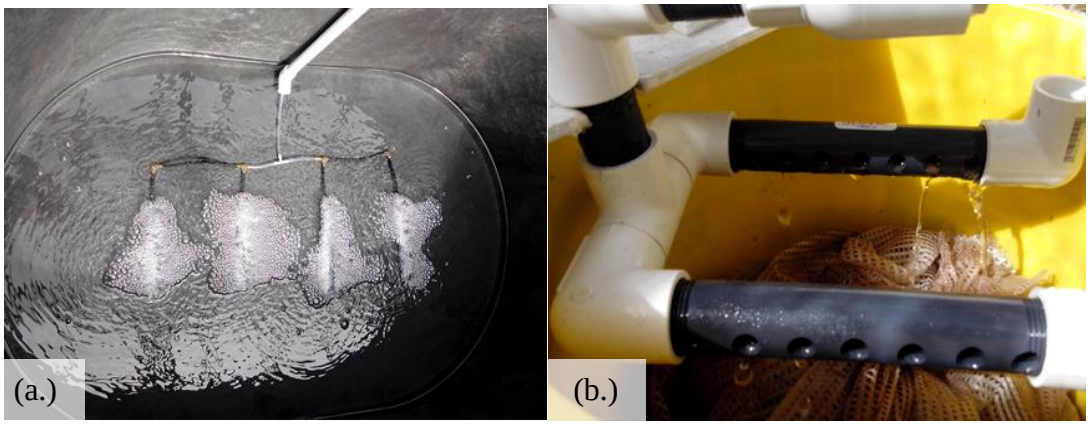

Figure 4.21. Aeration in fish tank (aerated surface) (a.) and aeration in downflow biofilter (biofilter 2) (non-aerated surface) (b.). Two aerating systems were considered as a cause of turbulent enhancing N_2O emissions in the aquaponics.

4.4.2. Development of strategies to reduce nitrous oxide emissions

Mitigations on N_2O emission reduction were developed with two strategies including (1) aerating an upflow aquaponic biofilter using an air diffuser (AA) and (2) adding soil effective microorganisms (EMs) (see section 3.2.4) to aquaponic systems once a week (AE) (see method details in section 3.2.4). Pak choi- and lettuce-based aquaponic systems operating at a feeding rate of 35 g/day and an HLR of 1.5 m^3/m^2-day were selected in this study. The hypotheses for two strategies and rationales of using the operating parameters were previously described in methodology section 3.2.4. Aquaponics with no additional mitigation (control) were compared with AA and EA in pak chi-and lettuce-based aquaponic systems.

The two strategies AA and AE did not prove to reduce N_2O emissions in aquaponic systems as hypothesized in the methodology. N_2O emissions and N_2O conversion ratios from aquaponic systems operated under the two strategies (AA and AE) in pak choi- and lettuce-based aquaponic systems were not significantly different from the aquaponics with no plants (Table 4.20). N_2O emissions were in ranges of 21.4 to 22.4 mg N/day and 22.8 to 25.1 mg N/day for pak choi and lettuce- based aquaponics, respectively (Table 4.20). Aeration in the upflow biofilter (AA) was a significant source of N_2O emissions besides nitrate generation (Table 4.20) although the aeration increased DO concentration in the biofilter. Plant species with the supplemental DO concentration (by aeration) and effective microorganisms did not reduce N_2O emissions. Plant nitrogen recovery efficiencies in pak choi- and lettuce-based aquaponics from the three systems were not significantly different. Nitrogen recovery in pak choi accounted for 15.7 ± 0.4%, 16.0 ± 0.3%, and 15.9 ± 0.7% for control, AA, and EM, respectively, and those in lettuce were accounted for 13.7 ± 0.3%, 13.8 ± 0.8%, and 13.4 ± 1.1% for control, AA, and EM, respectively. There were no effects of aeration and EMs on the plant growth and nitrogen recovery, leading to low effectiveness of using special aeration and EMs on N_2O emissions. These results agreed with N_2O emissions from other aquaponics that aeration was the major pathway of N_2O emission even DO concentration increased because N_2O could by stripped out by turbulence of air mixing and high DO levels could inhibit nitrous oxide reductase, which flavours anoxic condition (Hu et al., 2013, 2012).

The two strategies was not a promising mitigation to reduce N_2O emissions due to many unpractical challenges. In this study, aerating the up flow biofilters with air flow rate of 1.0

m^3/min increased DO concentrations at the bottom of the biofilter above 6 mg/L (measured on-site). However, this high DO levels did not reduce the total N_2O emissions due to the air turbulence and the inhibition of nitrous oxide reductase by high DO levels (Fang et al., 2017; Hu et al., 2013). EMs could not be an efficient mean to reduce N_2O emissions if carbohydrate or carbon sources (COD concentration) were not abundant for denitrifying bacteria at anoxic condition, leading to lack of electron donors for a complete denitrification (Hu et al., 2014). Another possibility associated to N_2O emission could be water quality in aquaponic systems (Hu et al., 2012). N_2O emission in aquaponics or aquaculture could be lowered if the systems were maintained at high C:N ratio and low nitrite concentrations (Hu et al., 2012; Wongkiew et al., 2017a).

Table 4.20. Nitrous oxide (N_2O) emissions and N_2O conversion ratios from pak choi and lettuce-based aquaponic systems with additional strategies to reduce N_2O emissions

Plant species	Strategies in aquaponics	Fish tank (mgN/day)	Biofilter 2 (mgN/day)	Biofilter 1 (upflow) (mgN/day)	N_2O emission (mgN/day)	N_2O conversion ratio (%)
Pak choi	Control	17.0 (2.5)[a]	4.5 (0.9)[a]	-	21.4 (2.4)[a]	0.89
	Aeration (AA)	17.4 (2.2)[a]	4.4 (0.2)[a]	0.4 (0.3)	22.2 (1.9)[a]	0.93
	EM (AE)	17.6 (4.3)[a]	4.8 (0.6)[a]	-	22.4 (4.4)[a]	0.93
Lettuce	Control	18.0 (3.1)[a]	4.8 (2.0)[a]	-	22.8 (1.2)[a]	0.94
	Aeration (AA)	18.4 (3.2)[a]	4.7 (2.3)[a]	2.0 (1.5)	25.1 (2.7)[a]	1.03
	EM (AE)	18.6 (2.7)[a]	4.7 (1.7)[a]	-	23.3 (1.8)[a]	0.96

(Note: Values are the mean of multiple data (n = 4), and values in parenthesis represent standard deviation. The superscripts a and b represent statistically different ($p < 0.05$))

TKN, TAN and COD concentrations and nitrate accumulation rates in aquaponics at control and AE conditions were not different (Table 4.21). However, nitrite concentrations in aquaponics at AA conditions were significantly lower than the control and AE conditions (Table 4.2.1), suggesting that high DO concentrations increased nitrite oxidation efficiency and subsequently reduce nitrite concentration. Although the lower nitrite concentrations were found in both pak choi- and lettuce-based aquaponic systems at the AA conditions, the N_2O emissions

from AA conditions were not significantly different from those in the control and AE aquaponics. Since nitrite is an intermediate of N_2O and nitrite concentration positively affect N_2O emissions rate (Wunderlin et al., 2012), the nitrite concentrations at AA condition might not be low enough to make a difference in N_2O emissions. Thus, N_2O emissions could be more affected by water quality rather than only aerating anoxic zone and amending external effective microbes to the aquaponic systems. Reducing anoxic zones and maintaining optimal feed-to-plant ratio (or feeding rate) could be other possibility to reduce N_2O emission from aquaponic systems (Hu et al., 2012). In the next section, N_2O emission rates at two feeding rates were compared to elucidate the discussion in this section.

4.4.3. Effect of feeding rates on nitrous oxide emissions

Feeding rate had significant impacts on N_2O emissions and water quality in aquaponic systems. Aquaponics operating at a high feeding rate of 35 g/day emitted higher N_2O than the aquaponics at feeding rate of 15 g/day, contributing for 0.84% and 0.75% of nitrogen input when feeding at 35 and 15 g/day, respectively (Table 4.22). Aquaponics operating at feeding rate of 15 g/day emitted N_2O of 7.8 mg N/d, which was significantly lower than aquaponics at feeding rate of 35 g/day by 62%. N_2O emissions from both biofilters and fish tanks in aquaponics operating at the feeding rate of 15 g/day were lower than those at the feeding rate of 35 g/day (Table 4.22). Fish tank, where aeration took place, was a major source of N_2O emission accounting for 69-76%. High N_2O emissions from fish tanks suggested that N_2O was generated in anoxic zone (e.g., biofilter and grow bed), but aeration had a higher influence to stripped out the dissolved N_2O to the atmosphere (Fang et al., 2017).

Table 4.21. Water quality parameters (nitrogen and COD concentrations, and NO_3^- accumulation rates) in pak choi- and lettuce-based aquaponic systems operated with strategies for N_2O emissions

Plant species	Strategies in aquaponics	TKN (mgN/L)	TAN (mgN/L)	NO_2^- (mgN/L)	NO_3^- accumulation rate* (mgN/L/d)	COD (mg/L)
Pak choi	Control	5.2 (0.8)	0.8 (0.1)	0.24 (0.04)[a]	0.74 (0.05)[a]	56.5 (6.9)[a]
	Aeration (AA)	5.0 (0.9)	0.9 (0.2)	0.17 (0.02)[b]	0.90 (0.12)[a]	56.2 (7.8)[a]
	EM (AE)	5.5 (1.1)	1.0 (0.2)	0.24 (0.03)[a]	0.78 (0.09)[a]	58.5 (6.9)[a]
Lettuce	Control	5.6 (1.1)	0.9 (0.2)	0.25 (0.03)[a]	0.84 (0.09)[a]	64.2 (6.9)[a]
	Aeration (AA)	5.5 (0.7)	0.8 (0.2)	0.17 (0.02)[b]	1.0 (0.02)[a]	59.9 (5.7)[a]
	EM (AE)	5.6 (0.5)	1.0 (0.2)	0.24 (0.4)[a]	0.74 (0.11)[a]	63.9 (4.9)[a]

(Note: Values are the mean of multiple data for TKN, TAN, NO_2^- and COD concentrations (n = 10 for pak choi and lettuce), NO_3^- accumulation rate (n = 2), DO concentrations (n = 37 for pak choi, n = 32 for lettuce). Values in parenthesis represent standard deviation. * NO_3^- accumulation rates were shown instead of the average values because NO_3^- concentrations did not reach steady state.)

Table 4.22. Nitrous oxide (N_2O) emissions and N_2O conversion ratios from lettuce-based aquaponic systems operating at feeding rates of 15 and 35 g/day

Lettuce-based aquaponics	Fish tank (mg N/day)	Biofilter (mg N/day)	N_2O emission (mg N/day)	N_2O conversion ratio (%)
Feeding rate: 35 g/day	15.6 (2.2)[a]	4.8 (1.0)[a]	20.4 (1.8)[a]	0.84
Feeding rate: 15 g/day	5.4 (1.1)[b]	2.4 (0.7)[b]	7.8 (0.7)[b]	0.75

(Note: Values are the mean of multiple data (n = 6), and values in parenthesis represent standard deviation. The superscripts a and b represent statistically different ($p < 0.05$))

Nitrite, TAN, and COD concentrations in the aquaponics at feeding rate of 15 g/day were lower than the aquaponics at 35 g/day (Table 4.23), suggesting that feeding rate also positively affected the water quality in aquaponic systems. Lower feeding rate also caused a lower nitrate accumulation rate and nitrite concentrations (Table 4.23), leading to a lower nitrogen loss via denitrification (Figure 4.22). This result of nitrogen loss is also comparable to section 4.2.2 (Figure 4.8) in which feeding rates of 35 and 50 g/day were compared. Since lower nitrogen loss was generated at the lower feeding rate (15 g/day), NUE in plants (a relative value), was higher in aquaponics at the lower feeding rate (Figure 4.2.2). However, plant growths between the two conditions were not significantly different (p=0.228), suggesting lower feeding rate did not reduce the vegetable productivity in aquaponics as long as maintaining sufficient nitrate concentration. This has to be noted that fish production (growth rate) decreased when lowering the feeding rate (growth rate at 15 g/day = 0.0125 g wet weigh/day; growth rate at 35 g/day = 0.0228 g wet weigh/day, $p < 0001$). Thus, designing a good fish-to-plant ratio was necessary to maintain high productivities of both fish and vegetable in aquaponic systems. These results suggested that balancing between input and outputs could be the promising strategies to increase NUE, reduce N_2O emissions from aquaponic systems, and provide an excellent water quality for fish, plants, and microbes.

Table 4.23. Water quality parameters (nitrogen and COD concentrations, and NO_3^- accumulation rates) in lettuce-based aquaponic systems operating at feeding rates of 15 and 35 g/day

Lettuce-based aquaponics	TKN (mgN/L)	TAN (mgN/L)	NO_2^- (mgN/L)	NO_3^- accumulation rate* (mgN/L/d)	COD (mg/L)
Feeding rate: 35 g/day	5.6 (0.8)	0.9 (0.2)[a]	0.22 (0.05)[a]	0.89 (0.11)[a]	62.7 (6.0)[a]
Feeding rate: 15 g/day	5.1 (0.8)	0.5 (0.1)[b]	0.04 (0.03)[b]	0.28 (0.06)[b]	50.5 (4.9)[b]

(Note: Values are the mean of multiple data for TKN, TAN, NO_2^- and COD concentrations (n = 15), NO_3^- accumulation rate (n = 3), DO concentrations (n = 32). Values in parenthesis represent standard deviation. * NO_3^- accumulation rates were shown instead of the average values because NO_3^- concentrations did not reach steady state.)

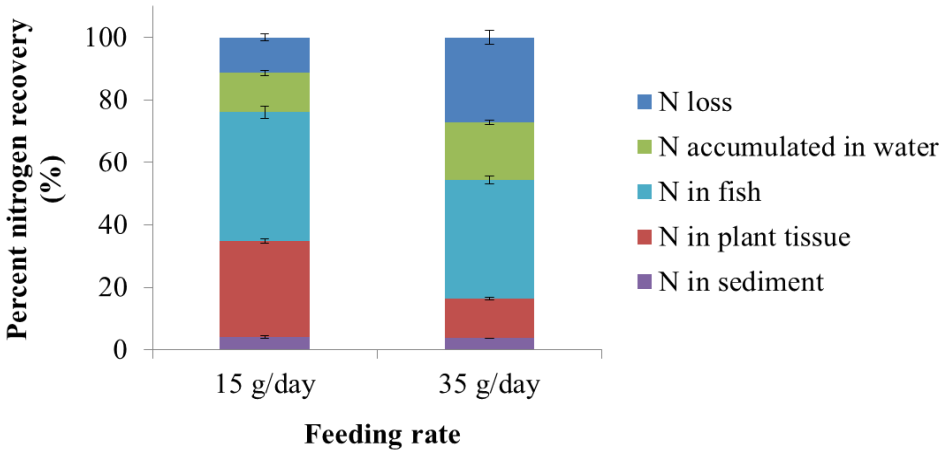

Figure 4.22. Nitrogen distribution of products in lettuce-based aquaponic systems operating at constant feeding rates of 15 and 35 g/day. Error bars represent the standard deviations of aquaponics operated in triplicate.

The two strategies in aquaponic systems were retested in aquaponics at feeding rate of 15 g/day. This task was done to ensure whether or not the effects of two strategies were not different when deruding feeding rate in the aquaponic systems. Results showed that even a lower feeding rate was applied in aquaponics, aerating the upflow biofilter and adding effective microorganisms did not efficiently reduce N_2O emissions (Table 4.24), and these strategies were not recommended for reducing N_2O emissions in floating-raft aquaponic systems. These results

supported to Tables 4.20 and 4.21 that only improving water quality by aerating the biofilter in aquaponics was not the first choice to reduce N_2O emissions because aeration enhanced the release of N_2O to the atmosphere. In conclusion, balancing feed-to-plant ratio was a recommended strategy to reduce N_2O emissions and nitrogen loss, and increasing NUE. Improving water quality by avoiding a large anoxic zone could help to reduce N_2O emissions, but aeration should not be used.

Table 4.24. Nitrous oxide (N_2O) emissions and N_2O conversion ratios from lettuce-based aquaponic systems operating at feeding rate of 15 g/day with additional strategies to reduce N_2O emissions

Strategies	**Fish tank (mgN/day)**	**Biofilter 2 (mgN/day)**	**Biofilter 1 (upflow) (mgN/day)**	**N_2O emission (mg N/day)**	**N_2O conversion ratio (%)**
Control	5.7 (1.0)^{a}	1.7 (0.9)$_{a}$	-	7.4 (0.4)^{a}	0.72
Aeration (AA)	5.4 (1.3)^{a}	1.4 (1.0)$_{a}$	0.8 (0.4)	8.1 (0.4)^{a}	0.78
EM (AE)	5.8 (0.9)^{a}	1.9 (0.7)$_{a}$	-	7.8 (0.3)^{a}	0.74

(Note: Values are the mean of multiple data (n = 4), and values in parenthesis represent standard deviation. The superscripts a and b represent statistically different ($p < 0.05$))

CHAPTER 5

GUIDLINES FOR NITROGEN RECOVERY

This study evaluated the nitrogen cycle, effects of operating parameters (e.g., DO, HLR, pH, and feeding) on nitrogen transformations, microbial community, and N_2O emissions from aquaponic systems. Balancing feed-to-plant was found to be the most economical way to reduce nitrogen loss, improve NUE, and reduce N_2O emission. Aeration that provides high DO concentrations (above 6 mg/L) in fish tank will be useful to maintain good water quality (low TAN and nitrite concentration) and slightly reduce nitrogen loss from aquaponic systems. Withdrawal of sediment from aquaponic biofilters at an interval of time could improve NUE because sediment created anoxic condition, which results in denitrification, leading to nitrogen loss. It was found that sediment in biofilters consisted of fish feces as a major portion, which resulted in the decrease in the abundance of nitrifying bacteria in the biofilters. Thus, regular withdrawal of sediment from biofilter or installing a sedimentation tank or pre-filtration/sedimentation unit before nitrification unit is recommended to improve NUE and nitrification in aquaponic systems. HLR associated the transfer of DO and nutrient to mix well in aquaponic systems. At a high HLR, farmers can ensure that aquaponic systems are well circulated throughout the aquaponic systems so that oxygen transfer did not limit ammonia and nitrite oxidation efficiency. Nitrite accumulation from a baseline nitrite level can indicate the insufficiency of HLR for a certain aquaponic systems. Nitrate accumulation and depletion can be used as the indicator to balance between nitrogen input and nitrogen outputs. Low pH levels (pH of 5.2) gradually increased TAN concentration in aquaponics, which will be harmful to fish.

Microbial community can be used as the key to understand the nitrogen transformations in different plant-based floating-raft aquaponic systems at a pH range of 5.2 to 7.0. This study elucidated the roles of microbial communities in nitrogen transformations in both plant roots and aquaponic biofilters (combined samples). It was found that plant species played an important role in improving nitrogen recovery. Although biofilters in aquaponic systems had the same function as existing nitrogen removal systems and agricultural soils (i.e., nitrification efficiency dropped at low pH levels), interestingly, plant roots in floating-raft aquaponic grow beds showed high abundances of nitrite-oxidizing bacteria even under acidic pH levels. The other benefit of plant

roots is that complete nitrification with a lower nitrogen loss via denitrification, in comparison to biofilters, could be achieved in the floating-raft grow beds without the need of biofilters if a total plant root surface area is sufficient for the microbial community. However, from engineering standpoint, biofilters should be installed in aquaponic systems to maintain a good buffering capacity for high nitrification and feces removal efficiency, and to prevent ammonia accumulations. Since the microbial structures in aquaponic systems are somewhat similar to existing biological nitrogen removal processes, the design approach may follow the same basic principles. This is one of the very first studies elucidating microbial structure in aquaponic systems for understanding nitrogen transformations and improving NUE. Overall, the study on the microbial communities suggested that, although pH dropped to acidic levels in aquaponic systems, growing plants with high root surface area, providing high DO for plant roots, and balancing nitrogen input and outputs, could enhance nitrification, thereby leading to the improvement in nutrient recovery in aquaponic systems as elucidated in Figure 5.1. Linking phosphorus transformations to microbial communities and studying legumes in aquaponic systems are recommended for future research to better understand another nutrient recovery and benefit more nitrogen inputs in aquaponic systems.

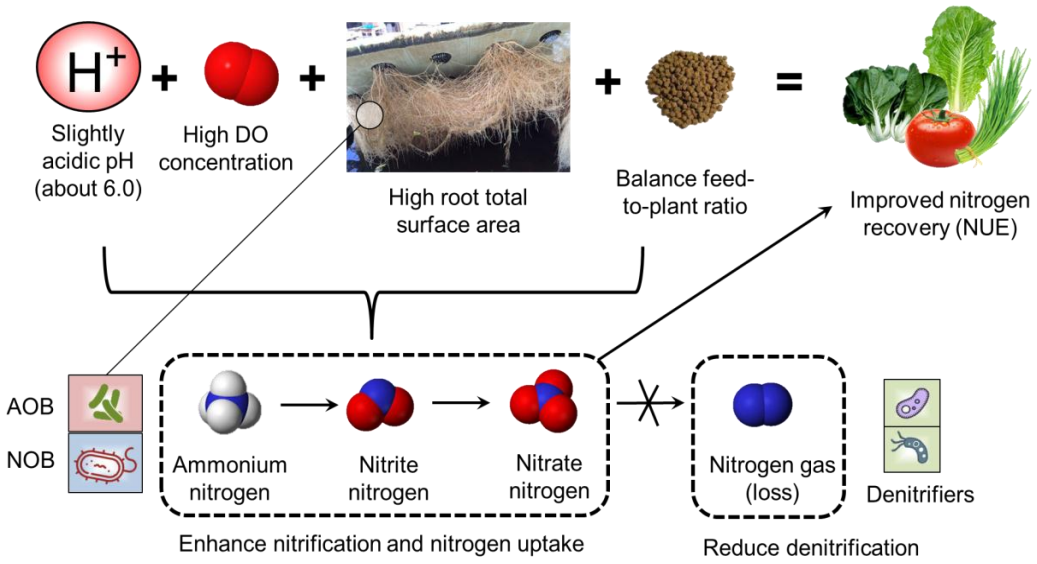

Figure 5.1. Strategies for improving nitrogen recovery in aquaponic systems by maintaining symbiotic environmental conditions for microbial community

CHAPTER 6

CONCLUSIONS

This study presented a comprehensive nitrogen cycle in floating-raft aquaponic systems. The investigation of the nitrogen cycle was conducted using the combinations of several research approaches such as nitrogen mass balance, natural abundance nitrogen isotope, enriched ^{15}N, 16S rRNA gene sequencing, qPCR, statistical analyses, and on-site samplings of nitrogen products. This study of the nitrogen cycle focused on four different aspects including (1) evaluating the operating parameters affecting nitrogen transformations in aquaponic systems for farmer's practical solutions, (2) understanding the fate and different forms of nitrogen for improving NUE, (3) understanding the ecology of microorganisms for further developments, and (4) reducing the environmental impact from N_2O emissions for future sustainable food production.

Key operating factors affecting nitrogen transformations in the aquaponic systems were evaluated. It was found that HLR, DO, pH, and feeding rate all played important roles in optimizing aquaponic systems. DO affected nitrite oxidation and denitrification in aquaponic systems. Nitrite concentrations increased at low DO level and low HLR. HLRs (0.10-0.25 m^3/m^2-day) did not have a direct effect on nitrogen transformations but associated mass transfer of oxygen and nitrogen for effective nitrification throughout aquaponic components, especially in biofilters. For example, significant increases of nitrite and TAN concentrations were found at HLRs of 0.25 and 0.1 m^3/m^2-day, respectively. An increase in nitrite concentration indicates a need to increase HLR or DO to reduce denitrification in the biofilter and to obtain a better water quality for fish. Low pH level of 5.2 caused the accumulation of TAN and drastically increased TAN concentrations in aquaponic systems. Stable TAN concentrations can be maintained at pH around 6.0 to 7.0, but TAN average concentrations at pH 7.0 was lower than 6.0. The growth of plant species was independent of HLR between 0.10 and 2.5 m^3/m^2-day. Reducing feeding rate in the presence of nitrate accumulation reduce nitrogen loss from aquaponic systems.

Based on nitrogen mass balance and natural abundance nitrogen isotopic compositions, where higher $\delta^{15}N$ values of nitrate relative to fish feed was found, denitrification occurred predominately at low DO level but was found to affect nitrogen loss under all DO levels. Based

on the isotopic mass balance of enriched ^{15}N, there were two pathways on nitrogen loss in aquaponic systems, namely nitrogen loss via a complete denitrification and nitrogen loss via direct reduction of nitrite after ammonia oxidation. The denitrification via direct reduction of nitrite was enhanced by fast-growing plant species, microbial community on large surface area of plant roots, low DO levels, and micro-anoxic environments in aquaponic systems. Nitrogen loss as N_2 through denitrification was able to be decreased by reducing feeding rate. However, maintaining a high DO level in fish tanks did not significantly decrease nitrogen loss. Reducing feeding rate decreased the nitrogen loss. Nitrate accumulation in recirculating water indicated that nitrate generation rate exceeded the nitrogen uptake rate by plants, while nitrate depletion suggested the insufficiency of nitrogen input for plant production. Efflux of nitrate from plant roots in the aquaponics indicated the incomplete assimilatory nitrate reduction in the plants. When the nitrate accumulation occurs, maintaining aerobic environment, maximizing plant-to-fish ratio, and regular withdrawal of sediment from biofilters are recommended to reduce the nitrogen loss from the aquaponic systems. Sediment in biofilters contained more than 90% of nitrogen from fish feces.

Low abundance of nitrifiers and high abundance of heterotrophic microorganisms in aquaponic components (e.g., plant roots and biofilters) supported the nitrogen loss, which decreased NUE in aquaponic systems. Low pH level was a major factor that shifted the microbial communities and reduced the relative abundance of nitrifiers in aquaponic components, leading to TAN accumulation in recirculating water. Microbial community compositions in plant roots and biofilter were distinct and have their specific compositions. However, the microbial communities in roots of lettuce, pak choi, tomato, and chive were similar to each other. Microbial community of fish intestinal track was dominant in biofilters while groups of NOB and rhizobacteria were found in plant roots. Interestingly, in plant roots, the abundance of major NOB (e.g., *Nitrospira* spp.) did not decrease at low pH levels, suggesting the benefit of growing plants in aquaponics for effective nitrification and improving NUE.

N_2O emissions from aquaponic systems were evaluated, and strategies to reduce N_2O were tested. Plant species (lettuce, pak choi, tomato, and chive) did not affect N_2O emissions despite their different root surface area. N_2O emissions about 20.4 - 25.1 mg N/day (0.8-1.0% of

total nitrogen loss) were found in aquaponic systems operating at HLR of 1.5 m^3/m^2-day and feeding rate of 35 g/day. Addition of EM and aerating biofilter did not reduce N_2O emissions from aquaponic systems because EM did not significantly improve the water quality and aeration stripped out dissolved N_2O from the recirculating water to the atmosphere. Reducing feeding rate from 35 g/day to 15 g/day significantly reduced the N_2O emissions and decreased TAN and nitrite concentrations but did not deplete nitrate concentration (rate of nitrogen input from feed was still higher than the rates of nitrogen uptake by plant). Results from this part suggested that lowering feeding rate or design aquaponics with a low fish-to-plant ratio could be the best strategies reduce N_2O emissions from aquaponic systems.

The outcomes of this study could provide better insights of a nitrogen cycle in aquaponic systems, practical guidelines to maintain high performance aquaponic systems, and useful information for developing and adopting a sustainable food production technology.

CHAPTER 7

FUTURE WORKS

A nitrogen cycle in floating-raft aquaponic systems were elucidated in this study. Although the outcomes of this study can provide the fate of nitrogen, the nitrogen processes, guidelines to improve NUE, and strategies to reduce the environmental impacts from aquaponic systems, many future works should be done to further develop the total performance of aquaponic systems. More research should be continued in order to push aquaponic systems commercialized around the world for the future before nutrient and water resources become limited. Main further studies are listed below.

- **Phosphorus recovery:** Aquaculture wastewater contains high concentrations of phosphorus. Similar to nitrogen, phosphorus in wastewater effluent can lead to eutrophication, algal bloom, and hypoxia in aquatic environments. However, phosphorus is a macronutrient for plants, and it can be recovered as same as nitrogen. Phosphorus also has many form including reactive phosphorus and total phosphorus. Therefore, phosphorus recovery and phosphorus cycle should be evaluated in aquaponic systems to enhance the overall performance and nutrient recovery in aquaponic systems.
- **Gene expressions and measurements of microbial reactions:** This study looked at 16S rRNA gene and specific genes, which are DNA based analyses. The results of this study (DNA) can review "who are there", termed microbial relative abundance, but not "what are they doing." Studying gene expression using the molecular analyses of mRNA via reverse transcriptase can review the activity of genes of interest in aquaponic systems. Gene expressions and measurements of microbial reactions can link the activities of genes in aquaponic systems with the microbial community in this study. This can give a better insights and lead to new conclusions of the nitrogen cycle in aquaponic systems.
- **Techno-economics analysis (TEA):** TEA is the tool that is used to evaluate the possibility of technology in term of costs-benefits and economic risks of a single technology. TEA can also be used to compare the economic quality of different technology before making a decision. Commercial farmers and governmental sectors need more information of TEA from aquaponics. TEA could help farmers to make

decisions to adopt aquaponics, and TEA can suggest governments to support aquaponic technology for alternative farming in the future. Thus, TEA of aquaponics in comparison with other vegetable production systems should be studied.

- **Life cycle assessment (LCA):** LCA relies on the concept of "cradle to gate" in which all resources used in the productions and extractions are included and analyzed. Since LCA bases on material flow analysis, LCA can focus on different boundaries of interest such as the infrastructure, energy use, water use, carbon footprint, fertilizers (soil organic farming), chemicals (hydroponics), and fish feed (aquaponics). LCA of aquaponic systems can help farmers to manage aquaponic systems and can help governmental sectors to evaluate the environmental impact and long-term sustainability of aquaponic systems. Thus, LCA of commercial aquaponic systems should be studied.
- **Nitrogen cycle in commercial-scale, NFT and flood-and-drain aquaponic systems:** In this study, only nitrogen cycle in pilot-scale floating-raft aquaponic systems was studied. However, NFT and flood-and-drain aquaponic systems have also been widely used in commercial scales. Nitrogen products distribution in different types and scales of aquaponic systems might be similar, but not identical, to the pilot-scale floating-raft aquaponic systems in this study. The different percent nitrogen recovery and nitrogen transformations could lead to different strategies to maximize NUE, reduce N_2O emissions, and increase aquaponic performance in aquaponic systems. Hence, nitrogen cycle in NFT and flood-and-drain aquaponic systems should be studied for a better connection among all types of aquaponic systems. Correlations between nitrogen transformations and operating conditions in each types of aquaponic systems and correlation between pilot-scale and large-scale aquaponics should be evaluated.
- **Integrations of aquaponic systems with another alternative input:** Protein-rich (40%) fish feed was used in this study. However, fish feed is produced from another protein source such as fish meat, resulting in a non-complete nitrogen recovery. To better recover nitrogen, another nitrogen input from other processed nitrogen-rich wastes such as waste-derived fungus, microalgae, macroalgae, and food residues from industries can be studied as a nitrogen input. Integration aquaponics with other farm wastes such as nitrogen waste

from poultry industries might be possible. Therefore, future works can focus on how to integrate aquaponics with other nitrogen inputs besides fish feed.

APPENDIX A

ANALYTICAL METHODS

Table A.1. Analytical methods and their identifications for determining nitrogen, iron and COD concentrations in this study

Parameters (concentrations)	Analytical Methods	Method IDs
Kjeldahl nitrogen (TKN)	HACH Digesdahl digestion	HACH 8075 (Loveland, CO, USA)
Total ammonia nitrogen (TAN)	Nessler	HACH 8038 (Loveland, CO, USA)
Nitrite nitrogen (NO_2^-)	NitriVer® 3 diazotization	HACH 8507 (Loveland, CO, USA)
Nitrate nitrogen (NO_3^-)	Dimethylphenol	HACH 10206 (Loveland, CO, USA)
Chemical oxygen demand (COD)	Reactor Digestion	HACH 8000 (Loveland, CO, USA)
Iron (Fe)	1, 10 Phenanthroline	HACH 10229 (Loveland, CO, USA)
TKN in stems, roots, leaves, sediment in biofilters, feed, and fish muscle tissue	HACH Digesdahl digestion followed by Nessler	HACH 8075 followed by HACH 8038 (Loveland, CO, USA)
Sediment concentration	Standard Methods	APHA, 2005

Table A.2. Quantitative polymerase chain reaction (qPCR) primer sets and details for quantifying copy numbers of eubacteria, ammonia oxidizing bacteria (AOB), ANAMMOX bacteria, *Nitrospira* spp., and *Nitrobacter* spp.

Target group	Primer/probe	Sequence (5'-3')	Ta(˚C)	References
Eubacteria	1055F	ATGGCTGTCGTCAGCT	53	Ferris et al. (1996)
	1395R	ACGGGCGGTGTGTAC		
AOB	*amoA*-1F	GGGGTTTCTACTGGTGGT	57	Rotthauwe et al. (1997)
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC		
ANAMMOX	Pla46F	GGATTAGGCATGCAAGTC	55	Van der Start et al. (2007)
	Anammox 667R	ACCAGAAGTTCCACTCTC		
***Nitrospira* spp.**	Nspra-675f	GCGGTGAAATGCGTAGAKATCG	58	Graham et al. (2007)
	Nspr-746r	TCAGCGTCAGRWAYGTTCCAGAG		
	Nspra-723Taq	CGCCGCCTTCGCCACCG		
***Nitrobacter* spp.**	Nitro1198f	ACCCCTAGCAAATCTCAAAAAACCG	58	Graham et al. (2007)
	Nitro1432r	CTTCACCCCAGTCGCTGACC		
	Nitro1374Taq	AACCCGCAAGGGAGGCAGCCGACC		

APPENDIX B

CALCULATION METHODS

Equations B1-B4. Nitrogen mass balance calculation

The nitrogen budget can be expressed as the rate of nitrogen changed during the period when the fish was fed (nitrogen input) until the products were harvested or generated (nitrogen outputs) as (see nitrate accumulation rates in Table 1):

$$f_N \times M_f = d/dt(C_{TAN} + C_{NO2\text{-}N} + C_{NO3\text{-}N} + C_{org\text{-}N})V + N_{veg}/T + N_{fish}/T + N_{sed}/T + N_{loss}/T \quad (B1)$$

The equation (S1) can be modified to an overall production-based mass balance equation.

In case of linear nitrate accumulation (lettuce, pak choi, chive (pH 6.0 to near neutral pH)):

$$f_N \times M_f \times T = (\text{nitrate accumulation rate}) \times V \times T + N_{veg} + N_{fish} + N_{sed} + N_{loss} \quad (B2)$$

In case of non-linear nitrate depletion (Tomato):

$$f_N \times M_f \times T = \int_0^T \text{nitrate accumulation rate (dt)} \times V + N_{veg} + N_{fish} + N_{sed} + N_{loss} \quad (B3)$$

In case of linear nitrate and TAN accumulations (chive (pH 5.2)):

$$f_N \times M_f \times T = (\text{nitrate + TAN accumulation rates}) \times V \times T + N_{veg} + N_{fish} + N_{sed} + N_{loss} \quad (B4)$$

Where, f_N is the fraction of nitrogen in fish feed (gN/g); M_f is the feeding rate (g/day); C_{TAN}, $C_{NO2\text{-}N}$, $C_{NO3\text{-}N}$ and $C_{org\text{-}N}$ are the TAN, nitrite, nitrate, and organic nitrogen concentrations in recirculating water (sampled from fish tank effluent) (gN/L), respectively; V is the volume of recirculating water (L); N_{veg} is the average nitrogen gained in vegetables at the end of each experiment (harvesting cycle) (gN); N_{fish} is the average nitrogen in fish muscle tissue (gN); T is time (days) for each harvest; N_{sed} is the nitrogen in sediment accumulated in biofilters at the end of each batch (gN); and N_{loss} is the gaseous nitrogen loss (gN). In this study, N_{loss} was unknown and was calculated by subtracting the nitrogen in fish feed (left side of the Eqs. B1 to B4) from the rest of known nitrogen products.

Equations B5-B6. Nitrogen isotope mass balance calculation

Mass of nitrogen loss from $^{15}NO_3^-$ was calculated using isotopic mass balance (Eq. B5), and mass of N loss from feed was calculated using nitrogen mass balance (Eqs. B1 to B4). Atom percent and nitrogen masses in Tables 4.11, 4.12, and 4.14 were used to calculate mass of nitrogen loss (an unknown). Since $^{15}NO_3^-$ was added to the systems, NO_3^- was considered as an input. There were two sources of nitrate inputs including daily nitrate generation from fish feed and nitrate existing in aquaponics before the operation. Nitrogen outputs included nitrate accumulated in the water, nitrogen in whole plants (organic N and nitrate N), nitrogen in plant roots, and sediment. The Eqs.B1 to B4 show the isotope mass balance that was used to determine mass of N loss from fish feed. It should be noted that nitrate in the recirculating water at the beginning of operation was the only ^{15}N enriched nitrogen source.

$$F_{NO3,before} \times Nmass_{NO3,before} + f_{NO3} \times F_{feed} \times Nmass_{feed} = F_{NO3,after} \times Nmass_{NO3,after} + F_{NO3,plant} \times Nmass_{NO3,plant} + F_{orgN,plant} \times Nmass_{orgN,plant} + F_{orgN,sed} \times Nmass_{orgN,sed} + F_{N2} \times Nmass_{N2} \quad (B5)$$

Where $F_{NO3,before}$ and $F_{NO3,after}$ are the atom percent (at%) of nitrate in the recirculating water at the beginning and the end of operation, respectively. F_{feed} is the atom percent of organic nitrogen in fish feed (0.3683 at%); $F_{NO3,plant}$, $F_{orgN,plant}$, and $F_{orgN,sed}$ are the atom percent (at%) of nitrate in plants, organic nitrogen in plants, and organic nitrogen in sediment, respectively. $Nmass_{NO3,before}$ and $Nmass_{NO3,after}$ are the masses of nitrate (gN) in the recirculating water at the beginning and the end of operation, respectively. $Nmass_{feed}$ is the mass of organic nitrogen in fish feed added to the system over the entire operation (gN); $Nmass_{NO3,plant}$, $Nmass_{orgN,plant}$, and $Nmass_{orgN,sed}$ are the masses of nitrate in plants, organic nitrogen in plants, and organic nitrogen in sediment at the end of operation (gN), respectively. F_{N2} is atom percent of nitrogen gas (at%) (average from the atom percent of nitrate in the water at the beginning and the end of operation). $Nmass_{N2}$ is the mass of nitrogen gas (unknown).

Percent nitrogen loss from nitrite reduction (%) was calculated as a percentage relative to mass of nitrogen loss from feed (gN) from nitrogen mass balance. Direct nitrogen loss from nitrite reduction (%) and nitrogen loss from nitrate reduction (%) were calculated using Eq. B6.

$$\text{N loss from nitrite reduction} = (\text{Mass N loss from } ^{15}NO_3^- / \text{ Mass N loss from feed}) \times 100 \quad (B6)$$

Table B.1. Calculation steps for determining the dose of added ^{15}N enriched compound to an aquaponic system (isotope addition)

Step no.	Information needed to calculate the dose of a spiked ^{15}N enriched compound to an aquaponic system	Value	Data/Equation
1	Baseline $\delta^{15}N$ value of a nitrogen form of interest in the recirculating water (‰)	A	A = input data
2	^{15}R of standard (air)	B	B = 0.003663/0.996337
3	Baseline ^{15}R value of the nitrogen form step line 1	C	C = ((A/1000)+1)*B
4	Fraction of ^{15}N (at%/100)	D	D = C/(1+C)
5	Fraction of ^{14}N (at%/100)	E	E = 1-D
6	Concentration of the nitrogen form of interest (mg N/L)	F	F = input data
7	Volume of the recirculating water (L)	G	G = input data
8	Mass of nitrogen (gN) in the recirculating water	H	H = F*G/1000
9	Mass of ^{14}N in the recirculating water (gN)	I	I = E*H
10	Mass of ^{15}N in the recirculating water (gN)	J	J = D*H
11	Multiplication factor to enrich ^{15}N abundance	K	H = input data
12	Mass of ^{15}N in the recirculating water after ^{15}N enrichment	L	L = J*K
13	$\delta^{15}N$ value of a nitrogen form of interest in the recirculating water after ^{15}N enrichment (‰)	M	M = (((L/I)/B)-1)*1000
14	Atom percent of the ^{15}N in a spiked compound (%at)	N	N = input data
15	Corrected $\delta^{15}N$ value of a nitrogen form of interest in the recirculating water after ^{15}N enrichment (‰)	O	O = ((L/((H+(1-N/100)*J))/B)-1)*1000
16	Molecular weight of the spiked compound (g/mol)	P	P = input data
17	% purity of the spiked compound (%)	Q	Q = input data
18	Number of N atom in a molecule of the spiked compound	R	R = input data
19	Mass of N per mole of the spiked compound (g/mol)	S	S = ((14*(100-N)/100) +(15*N/100))*R
20	Fraction of N in the spiked compound	T	T = S/P
21	Weight of the compound per bottle (g)	U	U = input data
22	Mass of N per bottle (g)	V	V = T*U
23	Mass of ^{15}N per bottle (g)	W	W = N/100*V
24	Fraction of single dose per bottle	X	X = (L-J)/W
25	Dose of the compound needed to spiked to the aquaponic systems (g)	Y	Y = X*V/(Q/100)

Table B.2. Example of the calculation steps for determining the dose of added ^{15}N enriched compound to an aquaponic system ($^{15}KNO_3^-$ as a spiked compound, isotope addition)

Step no.	Information needed to calculate the dose of a spiked ^{15}N enriched compound to an aquaponic system	Value	Data/Equation
1	Baseline $\delta^{15}N$ value of a nitrogen form of interest in the recirculating water (‰)	A	A = 15.4
2	^{15}R of standard (air)	B	B = 0.003663/0.996337
3	Baseline ^{15}R value of the nitrogen form in step 1	C	C = 0.0037331
4	Fraction of ^{15}N (at%/100)	D	D = 0.0037192
5	Fraction of ^{14}N (at%/100)	E	E = 0.9962808
6	Concentration of the nitrogen form of interest (mg N/L)	F	F = 100
7	Volume of the recirculating water (L)	G	G = 650
8	Mass of nitrogen (gN) in the recirculating water	H	H = 65
9	Mass of ^{14}N in the recirculating water (gN)	I	I = 64.758252
10	Mass of ^{15}N in the recirculating water (gN)	J	J = 0.241748
11	Multiplication factor to enrich ^{15}N abundance	K	H = 2
12	Mass of ^{15}N in the recirculating water after ^{15}N enrichment	L	L = 0.4834961
13	$\delta^{15}N$ value of a nitrogen form of interest in the recirculating water after ^{15}N enrichment (‰)	M	M = 1030.8
14	Atom percent of the ^{15}N in a spiked compound (%at)	N	N = 99
15	Corrected $\delta^{15}N$ value of a nitrogen form of interest in the recirculating water after ^{15}N enrichment (‰)	O	O = 1030.7
16	Molecular weight of the spiked compound (g/mol)	P	P = 102.1
17	% purity of the spiked compound (%)	Q	Q = 98
18	Number of N atom in a molecule of the spiked compound	R	R = 1 (R=2 for $(^{15}NH_4)_2SO_4$)
19	Mass of N per mole of the spiked compound (g/mol)	S	S = 14.99
20	Fraction of N in the spiked compound	T	T = 0.1468168
21	Weight of the compound per bottle (g)	U	U = 1
22	Mass of N per bottle (g)	V	V = 0.1468168
23	Mass of ^{15}N per bottle (g)	W	W = 0.1453487
24	Fraction of single dose per bottle	X	X = 1.6632282
25	Dose of the compound needed to spiked to the aquaponic systems (g)	Y	Y = 1.6971717

Equations B7-B14. Equations for modeling two extremes of natural abundance nitrogen isotopic compositions ($\delta^{15}N$) in the absence of denitrification and in the absence of fish feed

Nitrogen mass balance in aquaponics with no plants can be expressed as (B7).

$$d/dt\ (C_{NO3\text{-}N})_{,\ measured} = ((f_N.M_f - N_{fish}/T - N_{sed}/T) - N_{loss}/T)/V \qquad (B7)$$

Eq. (B8) expresses nitrate accumulation rate in the absence of denitrification which means that all of the nitrogen feed is converted to products. The nitrate accumulation in the absence of denitrification as a function of time was modeled using the same values as measured.

$$d/dt\ (C_{NO3\text{-}N})_{,\ absence\ of\ denitrification} = (f_N.M_f - N_{fish}/T - N_{sed}/T)/V \qquad (B8)$$

The rate of nitrogen loss can be calculated by subtracting actual nitrate accumulation rate from nitrate accumulation rate in the absence of denitrification. If nitrogen loss was attributed to the nitrogen loss via denitrification (B9), then the slopes should be less positive than the nitrate accumulation rate in the absence of denitrification ($N_{loss}/T = 0$ gN/day).

$$N_{loss}/T = (d/dt(C_{NO3\text{-}N})_{,\ absence\ of\ denitrification} - d/dt(C_{NO3\text{-}N})_{,\ measured})V \qquad (B9)$$

The decrease in nitrate with no feed in the presence of actual denitrification can be expressed by (B10) with the hypothesis that all nitrate will be converted over the time to nitrogen gas via denitrification. Nitrogen loss via denitrification was calculated from Eq. (B10).

$$(d/dt(C_{NO3\text{-}N})_{,\ no\ feed})V = -N_{loss}/T \qquad (B10)$$

Substituting Eq.(B10) into Eq. (B9), nitrate concentrations in the absence of feed can be expressed by (B11).

$$d/dt(C_{NO3\text{-}N})_{,\ no\ feed} = -(d/dt(C_{NO3\text{-}N})_{,\ absence\ of\ denitrification} - d/dt(C_{NO3\text{-}N})_{,\ measured}) \qquad (B11)$$

Isotope mass balance in the absence of denitrification can be calculated as follow:

$$[(\dot{m}_{feed}).(\Delta).(\delta^{15}N_{feed}) + (m_{NO3^-}).(\delta^{15}N_{NO3^-})]_{t=t} = [(m_{NO3^-}).(\delta^{15}N_{NO3^-}) + (\dot{m}_{fish}).(\Delta).(\delta^{15}N_{fish}) + (\dot{m}_{sed}).(\Delta).(\delta^{15}N_{sed})]_{t=t+\Delta} \qquad (B12)$$

Where $\dot{m}_{feed}$, $\dot{m}_{fish}$ and $\dot{m}_{sed}$ represent feeding rate, fish muscle growth rate and sediment generation rate (gN/d), t represents the duration (days) of the experiment 0, 3, 6, 9 and 12, Δ represents the time interval (3 days). The $\delta^{15}N_{feed}$, $\delta^{15}N_{fish}$ and $\delta^{15}N_{sed}$ (‰) represent the bulk $\delta^{15}N$ of feed, fish muscle tissue and sediment, respectively. These values were assumed to be constant every day for 12 days. The initial $\delta^{15}N_{NO3^-}$ values were assumed to be the same level as the actual values. Nitrate mass (g N) in recirculating water (m_{NO3^-}) at each sampling interval were obtained from the modeled nitrate concentrations (Figure 4.1).

In the absence of feeding, fish growth was assumed to be negligible. According to Eq. (3.5), an isotope effect (B13) was substituted to (B14) to solve the equation.

$$[(m_{NO3^-}).(\delta^{15}N_{NO3^-})]_{t=t} = [(m_{NO3^-}).(\delta^{15}N_{NO3^-}) + (\dot{m}_{sed}).(\Delta).(\delta^{15}N_{sed}) + (N_{loss}/T).(\Delta).(\delta^{15}N_{gas})]_{t=t+\Delta} \quad \text{(B13)}$$

$$\delta^{15}N_{gas} = \{(\delta^{15}N_{NO3^-} + 1000)/[(\varepsilon_{(NO3\text{-}gas)}/1000) + 1]\} - 1000 \quad \text{(B14)}$$

Where $(\dot{m}_{gas}).(\Delta)$ represents the total amount of gas loss (gN) over a time interval of 3 days. $\delta^{15}N_{gas}$ represent isotopic composition of nitrogen loss (‰) via denitrification, which was calculated based on the $\varepsilon_{(NO3\text{-}gas)}$ of 30 ‰ (Robinson, 2001).

APPENDIX C

OTHER MICORBIAL RESULTS OF 16S rRNA GENE SEQUENCING

Table C.1. Operational parameters and system performances referring to microbial samples (section 4.3) in pak choi-, lettuce-, tomato-, and chive-based aquaponic systems (neutral pH, pH of 6.0, and pH of 5.2)

Plant types	DO in fish tank (mg/L)	pH	TKN (mgN/L)	TAN (mgN/L)	NO_2^- (mgN/L)	NO_3^- accumulation rate (mgN/L/d)*	Range of NO_3^- (initial - final) (mg N/L)	COD (mg/L)
Pak choi	7.4 (0.3)	6.8 (0.2)	4.2 (1.4)	1.1 (0.2)	0.25 (0.07)	0.82	150 - 180	76.3 (5.5)
Lettuce	6.9 (0.2)	6.9 (0.1)	8.0 (1.5)	0.7 (0.1)	0.24 (0.06)	0.57	211 - 229	56.1 (2.9)
Tomato	6.8 (0.3)	7.0 (0.2)	7.8 (1.4)	0.9 (0.1)	0.15 (0.05)	$6.9(10^{-4})t^2$ - 0.11t+ 2.4	139 - 60	69.2 (6.9)
Chive	6.9 (0.2)	7.0 (0.4)	7.9 (1.7)	0.8 (0.2)	0.20 (0.05)	1.63	143 - 249	50.3 (5.9)
Chive 6.0	6.8 (0.4)	6.0 (0.2)	8.5 (1.8)	2.6 (0.6)	0.13 (0.04)	1.33	177 - 218	65.1 (5.6)
Chive 5.2	7.2 (0.4)	5.2 (0.2)	10.1 (2.4)	6.6 (5.2)	0.07 (0.03)	0.79	143 - 168	49.1 (3.1)

(Note: Values are the mean of multiple data for TKN, TAN, NO_2^- and COD concentrations (n = 15 for pak choi and lettuce, n= 36 for tomato, n= 30 for chive, n= 15 for chive pH 6.0 and pH 5.2), NO_3^- accumulation rate (n = 3), DO concentrations (n = 37 for pak choi, n = 32 for lettuce, n= 90 for tomato, n= 61 for chive, n = 29 for chive pH 6.0, n= 32 for chive pH 5.2). Values in parenthesis represent standard deviation. * NO_3^- accumulation rates were shown instead of the average values because NO_3^- concentrations did not reach steady state.)

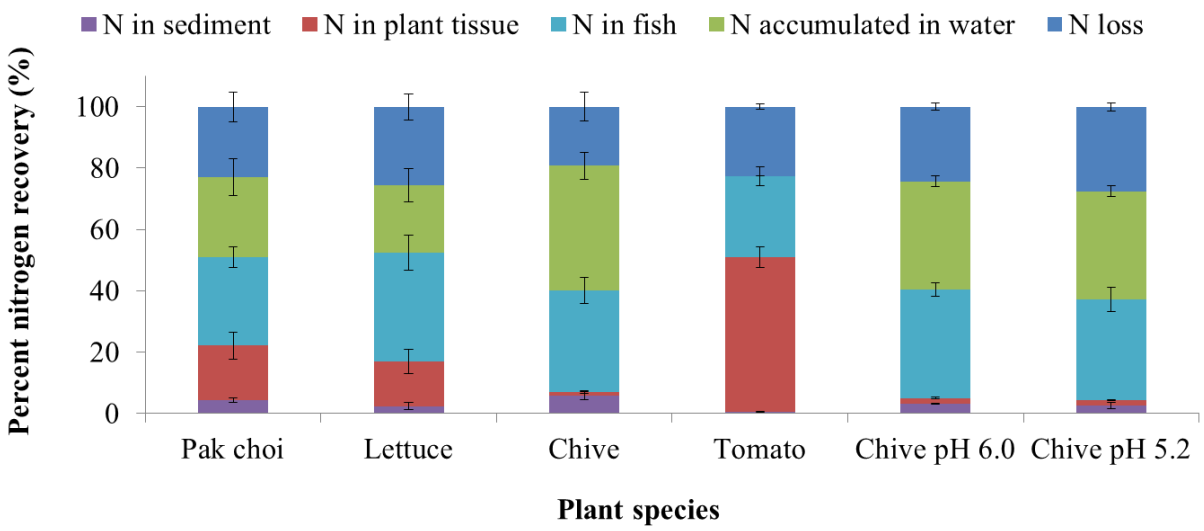

Figure C.1. Nitrogen distribution of products referring to microbial samples (section 4.3) in pak choi-, lettuce-, tomato-, and chive-based aquaponic systems (neutral pH, pH of 6.0, and pH of 5.2). Error bars represent the standard deviations of aquaponics operated in triplicate

Table C.2. Microbial diversity indices from combined (fish tank effluent, sediment, and biofilms) and root samples from chive-based aquaponic systems operated at pH 7.0 (Chive), 6.0 (Chive pH 6.0), and 5.2 (Chive pH 5.2)

Sample	Reads	Richness	Evenness	Shannon's diversity index	Simpson's index of diversity	Chao1	ACE	Good's coverage
Chive (combined)	46251	502	0.73	4.56	0.976	567	565	0.998
Chive pH 6.0 (combined)	5364	306	0.76	4.34	0.970	406	395	0.983
Chive pH 5.2 (combined)	18218	350	0.70	4.14	0.968	463	441	0.995
Chive (root)	20361	414	0.71	4.29	0.958	501	487	0.997
Chive pH 6.0 (root)	214	65	0.87	3.60	0.960	94	114	0.854
Chive pH 5.2 (root)	27291	330	0.68	3.92	0.959	405	415	0.997

Table C.3. Bacterial phyla as "other phyla" (Figure 4.16) and symbols (characters a to z, A1, and A2) representing "other phyla" in CCA plots from combined samples (fish tank effluent, sediment, and biofilms) (Figure 4.17) and root samples (Figure 4.18)

Symbols in Figures 4.17 and 4.18	Phyla (combined samples)	Phyla (root samples)
a	Armatimonadetes	uncultured
b	uncultured	BRC1
c	BRC1	Chlorobi
d	Cyanobacteria	Cyanobacteria
e	Deinococcus-Thermus	Deinococcus-Thermus
f	Elusimicrobia	Elusimicrobia
g	Eukaryote_unclassified	Eukaryote_unclassified
h	FBP	FBP
i	Firmicutes	Fibrobacteres
j	Gracilibacteria	Firmicutes
k	Hydrogenedentes	Gracilibacteria
l	Ignavibacteriae	Hydrogenedentes
m	Latescibacteria	Latescibacteria
n	Lentisphaerae	Microgenomates
o	Microgenomates	Omnitrophica
p	Omnitrophica	Parcubacteria
q	Parcubacteria	Peregrinibacteria
r	Peregrinibacteria	Spirochaetae
s	Saccharibacteria	Thaumarchaeota
t	Spirochaetae	TM6_(Dependentiae)
u	Tenericutes	unknown
v	Thaumarchaeota	Woesearchaeota_(DHVEG-6)
w	TM6_(Dependentiae)	WS2
x	unclassified	WS6
y	unknown	WWE3
z	Woesearchaeota_(DHVEG-6)	
A1	WS6	
A2	WWE3	

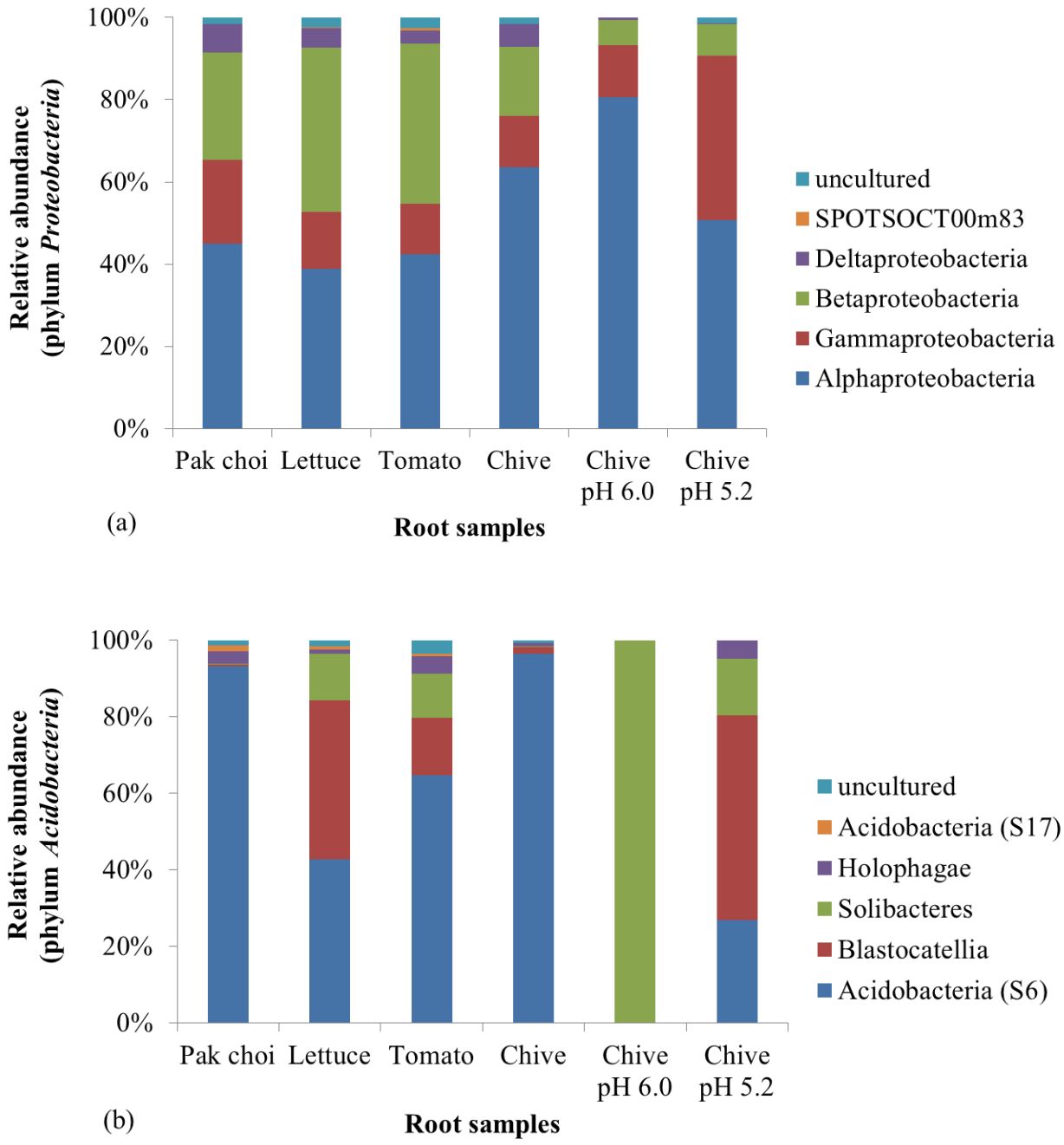

Figure C.2. Relative abundance of bacterial classes in phylum *Proteobateria* (a) and genus in phylum *Fusobacteria* (b) from combined samples (fish tank effluent, sediment, and biofilms) of different plant-based aquaponic systems and pH levels

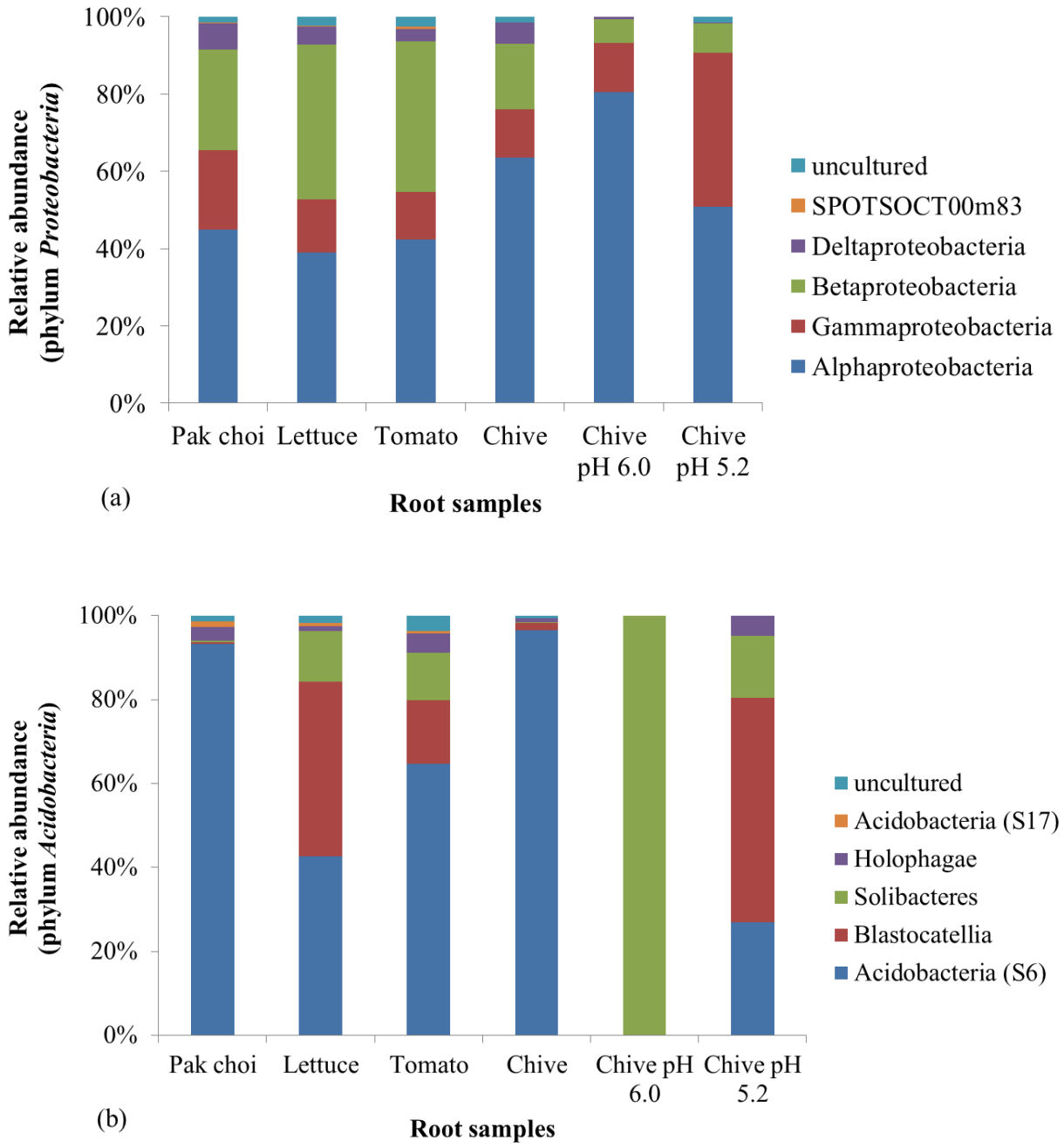

Figure C.3. Relative abundance of bacterial classes in phylum *Proteobateria* (a) and phylum *Acidobacteria* (b) from root samples of different plant-based aquaponic systems and pH levels

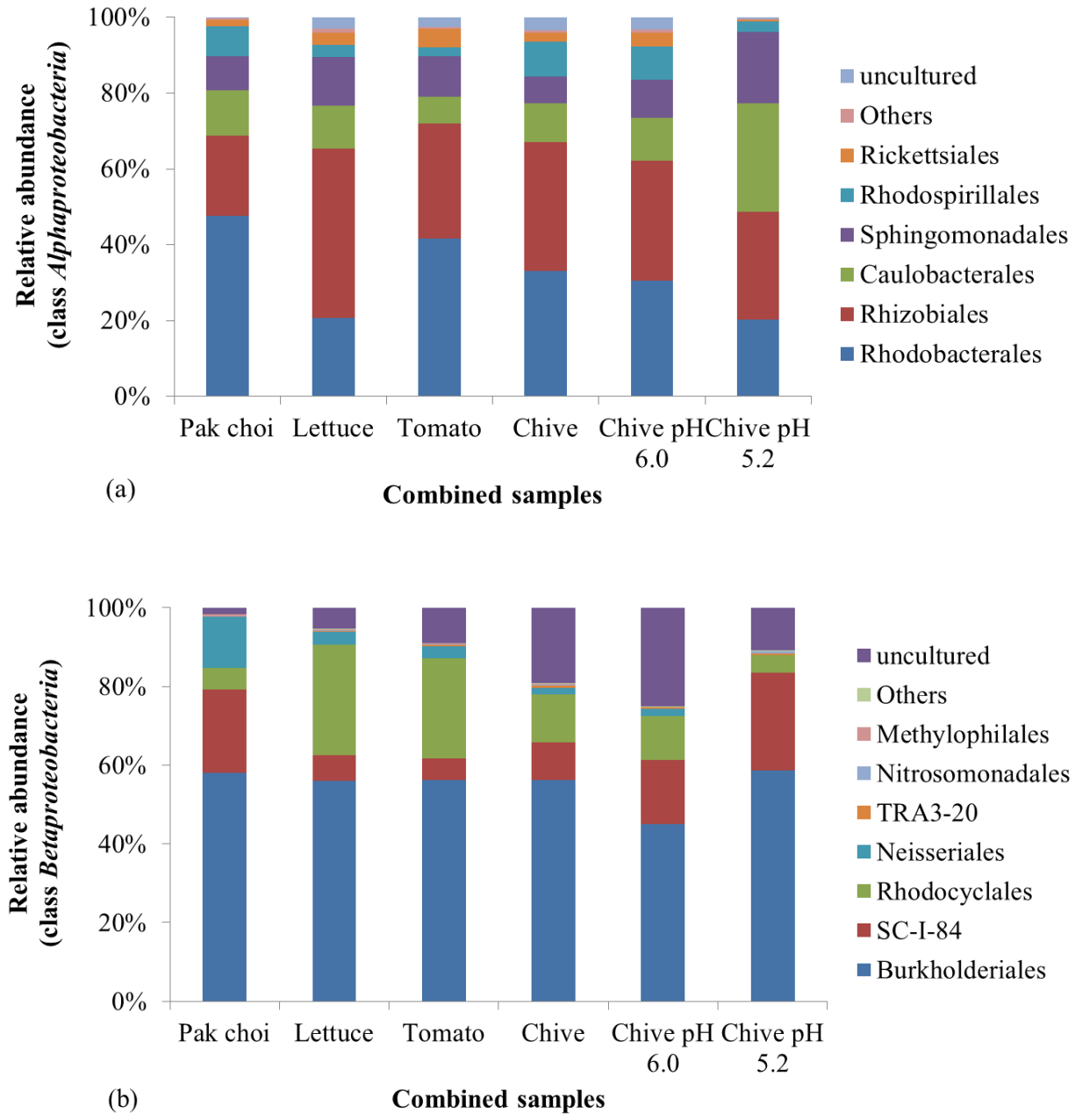

Figure C.4. Relative abundance of bacterial orders in class *Alphaproteobateria* (a) and class *Betaproteobacteria* (b) from combined samples (fish tank effluent, sediment, and biofilms) of different plant-based aquaponic systems and pH levels

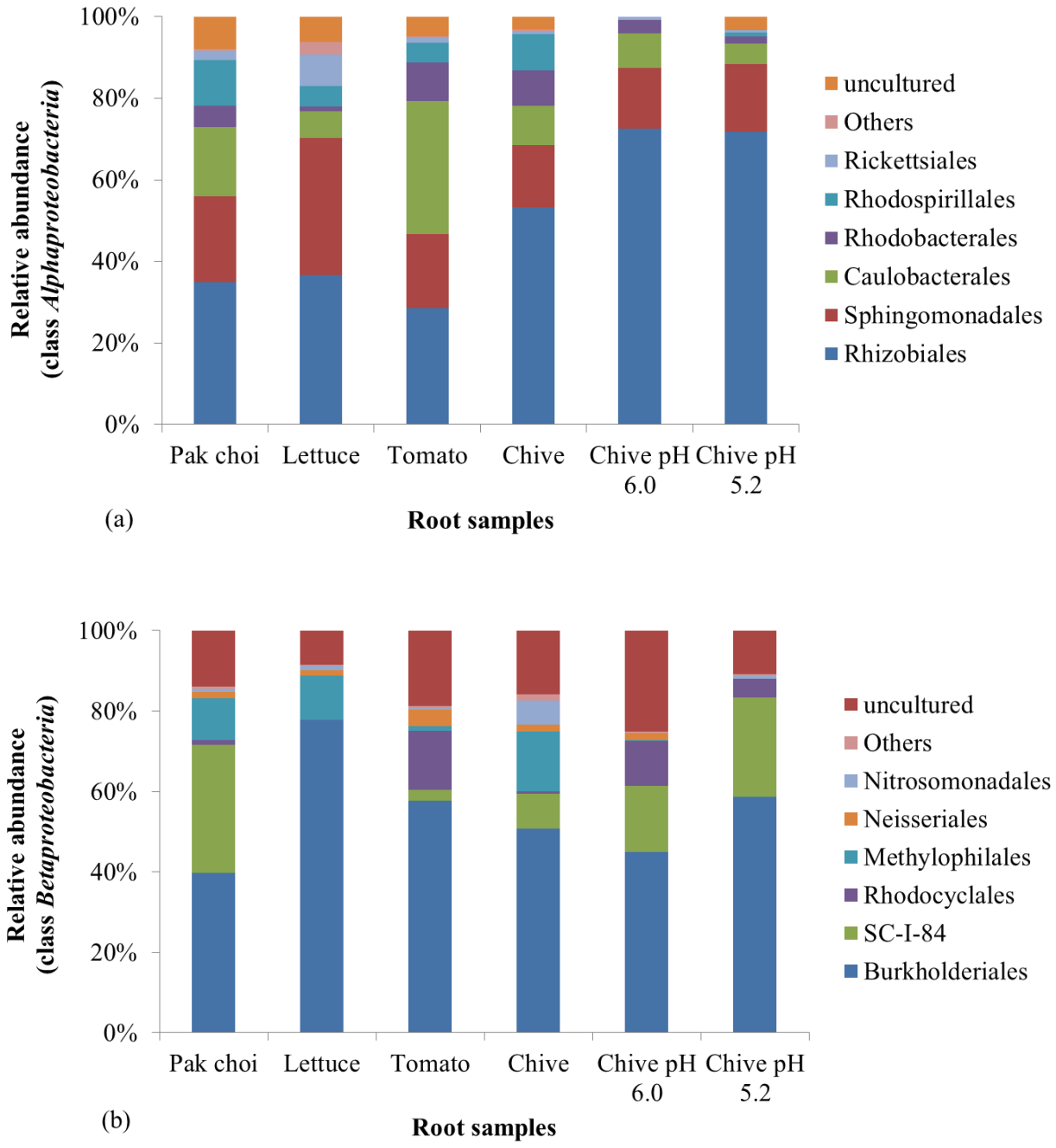

Figure C.5. Relative abundance of bacterial orders in class *Alphaproteobateria* (a) and class *Betaproteobacteria* (b) from root samples of different plant-based aquaponic systems and pH levels

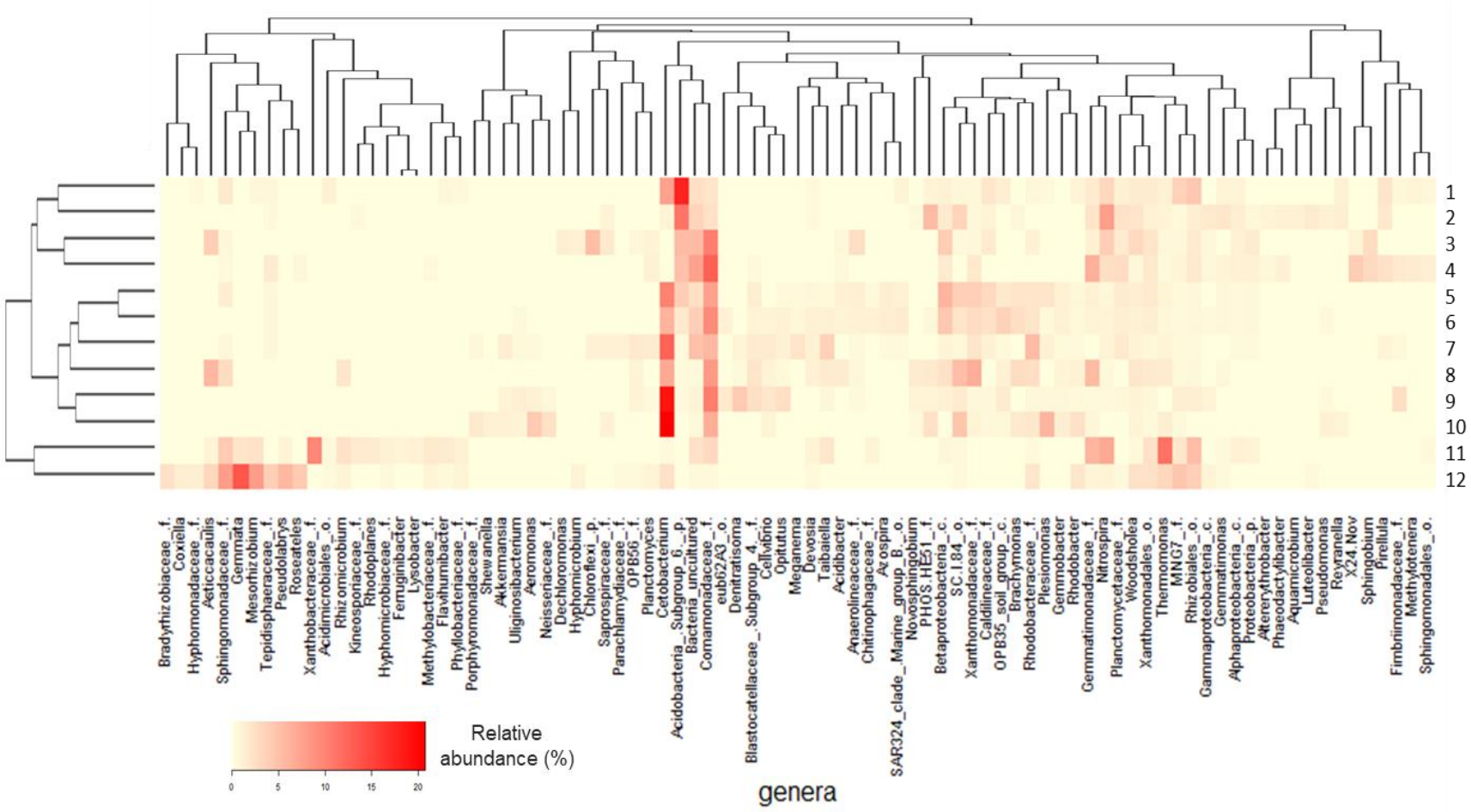

Figure C.6. Heatmap of microbial community compositions (87 OTUs) (genera, family (.f.), class (.c.), order (o.), and phylum (.p.)) and dissimilarities (Bray-Curtis) among all samples 1-12: (1) chive root, (2) pak choi root, (3) tomato root, (4) lettuce root, (5) chive pH 6.0 combined, (6) chive combined, (7) chive pH 5.2 combined, (8) tomato combined, (9) lettuce combined, (10) pak choi combined, (11) chive pH 5.2 root, and chive pH 6.0 root. All OTUs with relative abundance > 1% were included in this heatmap.

APPENDIX D

STATISTICAL ANALYSES OF qPCR RESULTS

Table D.1. Post-hoc Tukey's test of relative abundances of *Nitrospira* spp. genes in each sample at near neutral pH

Specific genes of interest, sample	Grouping	Mean value (copies/10^6 Eub copies)
Nitrospira spp., Sediment	A	207166
Nitrospira spp., Pak choi root	B	153100
Nitrospira spp., Tomato root	B	139250
Nitrospira spp., Chive root	C	49460
Nitrospira spp., Lettuce root	C D	46089
Nitrospira spp., Fish tank effluent	C D	9193.1
Nitrospira spp., Biofilm	D	848

Note: Different letters in grouping column represent significant different ($p < 0.05$)

Table D.2. Post-hoc Tukey's test of relative abundances of *amoA* genes in each sample

Specific genes of interest, sample	Grouping	Mean value (copies/10^6 Eub copies)
amoA, Biofilm	A	614.4
amoA, Fish tank effluent	B	210.4
amoA, Fish tank effluent (pH 5.2)	C	13.6
amoA, Biofilm (pH 6.0)	C	8.6
amoA, Fish tank effluent (pH 6.0)	C	8.6
amoA, Biofilm (pH 5.2)	C	6.9

Note: Different letters in grouping column represent significant different ($p < 0.05$)

Table D.3. 2-sample Student's *t* tests of relative abundances of *Nitrobacter* spp. genes in sediments at low vs. near neutral pH levels

Specific genes of interest, sample	*p* value
Nitrobacter spp., Sediment (pH 5.2) > *Nitrobacter* spp., Sediment	0.028
Nitrobacter spp., Sediment (pH 6.0) > *Nitrobacter* spp., Sediment	0.018

Note: Significant when $p < 0.05$

Table D.4. 2-sample Student's *t* tests of relative abundances of *Nitrobacter* spp. genes in chive roots at low vs. near neutral pH levels

Specific genes of interest, sample	*p* value
Nitrobacter spp., Chive root (pH 5.2) > *Nitrobacter* spp., Chive root	0.023
Nitrobacter spp., Chive root (pH 6.0) > *Nitrobacter* spp., Chive root	0.036

Note: Significant when $p < 0.05$

Table D.5. Post-hoc Tukey's test of relative abundances of *amoA* genes in root samples

Specific genes of interest, sample	Grouping	Mean value (copies/10^6 Eub copies)
amoA, Chive root	A	1080.9
amoA, Lettuce root	B	542.5
amoA, Pak choi root	C	196.7
amoA, Chive root (pH 6.0)	C	143.7
amoA, Chive root (pH 5.2)	C	120.9
amoA, Tomato root	C	83.3

Note: Different letters in grouping column represent significant different ($p < 0.05$)

Table D.6. Post-hoc Tukey's test of relative abundances of *Nitrospira* spp. genes in root samples

Specific genes of interest, sample	Grouping	Mean value (copies/10^6 Eub copies)
Nitrospira spp., Pak choi root	A	153100
Nitrospira spp., Tomato root	A B	139250
Nitrospira spp., Chive root (pH 5.2)	B C	89747
Nitrospira spp., Chive root (pH 6.0)	C	80250
Nitrospira spp., Chive root	C	49460
Nitrospira spp., Lettuce root	C	46089

Note: Different letters in grouping column represent significant different ($p < 0.05$)

Table D.7. Post-hoc Tukey's test of relative abundances of *Nitrobacter* spp. genes in root samples

Specific genes of interest, sample	Grouping	Mean value (copies/10^6 Eub copies)
Nitrobacter spp., Chive root (pH 6.0)	A	17249
Nitrobacter spp., Chive root (pH 5.2)	B	4840
Nitrobacter spp., Pak choi root	B C	927.3
Nitrobacter spp., Tomato root	B C	381.8
Nitrobacter spp., Chive root	B C	351
Nitrobacter spp., Lettuce root	C	243.5

Note: Different letters in grouping column represent significant different ($p < 0.05$)

APPENDIX E

LIST OF PUBLICATIONS

Peer-Reviewed Articles

Wongkiew, S., Hu, Z., Chandran, K., Lee, J.W., and Khanal, S.K. (2017). Nitrogen transformations in aquaponic systems: A review. *Aquacultural Engineering*, 76, 9-19.

Wongkiew, S., Popp, B.N., Kim, H.J., and Khanal, S.K. (2017). Fate of Nitrogen in Floating-Raft Aquaponic Systems using Natural Abundance Nitrogen Isotopic Compositions. *International Biodeterioration & Biodegradation*, 125, 24-32.

Wongkiew, S., Park, M.R., Chandran, K., and Khanal, S.K. Aquaponic Systems for Sustainable Resource Recovery: Linking Nitrogen Transformations to Microbial Communities. *Environmental Science and Technology* (submitted with major revision).

International conference

Wongkiew, S., Park, M.R., Popp, B.N., Chandran, K., and Khanal, S.K. Aquaponic System - An Emerging Technology for Resource Recovery, Oral presentation, The 2nd International Resource Recovery Conference, Columbia University, New York, August 5-9, 2017.

Oral & poster presentations

Wongkiew, S., and Khanal, S.K. A life story of nitrogen in aquaponics for resource recovery: Insights from molecular perspectives. Poster Presentation. 30th Annual College of Tropical Agriculture and Human Resources (CTAHR) and College of Engineering (COE) Student Research Symposium, University of Hawaii at Manoa, April 6-7, 2018.

Wongkiew, S., and Khanal, S.K. Nitrogen transformations in floating-raft aquaponic systems. Poster Presentation. 28th Annual College of Tropical Agriculture and Human Resources (CTAHR) and College of Engineering (COE) Student Research Symposium, University of Hawaii at Manoa, April 8-9, 2016.

Wongkiew, S., and Khanal, S.K. Nitrogen transformations in aquaponic systems. Oral Presentation. 27th Annual College of Tropical Agriculture and Human Resources (CTAHR) and College of Engineering (COE) Student Research Symposium, University of Hawaii at Manoa, April 10-11, 2015.

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